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THE EFFECT OF PARATHORMONE ON BASAL METABOLISM OF NORMAL DOGS

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There is a rather widely current view that vitamin D exercises its effects through the agency of the parathyroids. Evidence against this view has been presented by Harris and his co-workers (1) and by Dale, Marble and Marks (2). It was shown by Reed and Seed (3) that post-operative tetany may be controlled by means of activated ergosterol without significant alteration of blood calcium and also that some subjects responded to this treatment who were refractory to parathyroid extract.

Also, it has been shown (4) that activated ergosterol in the form of viosterol will increase the metabolic rate of normal dogs. It was believed that additional evidence for or against the involvement of the parathyroids in vitamin D functioning could be derived by determining whether parathyroid extract would affect the metabolic rate in a manner comparable to viosterol.

Five normal dogs were selected and trained as described in the earlier paper (4).

Adult female dog, 11.8 kilos body weight. After a training period the experiment was begun on February 16. During a control period up to March 1 the metabolic rate fluctuated between 940 and 1000 cal/M²/24 hrs. From March 1 to March 8 inclusive 4 cc. of parathormone[1] were injected intravenously each day. On March 10 the dose was increased to 6 cc. daily over 3 days. From March 14 to March 17 inclusive the daily dose was 10 cc. The metabolic rates determined during this period were as follows:

Mar. 1.....	930	Mar. 8.....	1000	Mar. 15.....	965	Mar. 22.....	970
Mar. 2.....	1020	Mar. 10.....	1000	Mar. 16.....	945	Mar. 23.....	1010
Mar. 3.....	950	Mar. 11.....	970	Mar. 18.....	920	Mar. 26.....	965
Mar. 5.....	975	Mar. 12.....	1000	Mar. 19.....	970	Mar. 28.....	918
Mar. 7.....	950	Mar. 14.....	965	Mar. 21.....	960		

[1] The parathormone was supplied through courtesy of Eli Lilly & Company.

Young female dog, 10.6 kilos. During 21 days' control observation the average rate was 880 cal/M²/24 hrs. with a maximum deviation of 17.6 per cent. Beginning on the 22nd day 1 cc. of parathormone was injected daily over 4 days. Over the next 9 days the daily dose was doubled. Over 8 days the dose was increased to 3 cc., to 4 cc. over 3 days, and finally to 8 cc. over 4 days, making a total of 89 cc. of parathormone.

The average rate for the 27 days of parathormone administration was 871 with a maximum deviation of ±11.7 per cent. There was, thus, no significant alteration in the rate.

Young male dog, 10 kilos. Training period 52 days; control period 10 days. Average rate, 974 cal/M²/24 hrs., ±5 per cent. One cubic centimeter of parathormone was injected daily for 3 days, 2 cc. daily for 3 days, and 9 cc. daily for 6 days. The average rate was 940 cal/M²/24 hrs., ±15 per cent for this period.

Adult male dog, 10.2 kilos. Training period 55 days, control period 19 days, with an average rate of 889 cal/M²/24 hrs., ±13.4 per cent. On June 1, 1 cc. of parathormone was injected, 2 cc. daily over 3 days, 3 cc. daily over 4 days, 4 cc. daily over 7 days, 8 cc. daily over 4 days, and a final single injection of 10 cc. which resulted in death of the animal during the succeeding night. The average rate for the entire period of administration was 742 cal/M²/24 hrs., ±28 per cent.

Young male dog, 9.9 kilos. Training period 31 days; control period, 6 days; average rate 871 cal/M²/24 hrs., ±13.8 per cent. During 5 days, 4 cc. of parathormone were injected daily. During this period the average rate was 795 cal/M²/24 hrs., ±10 per cent.

These experiments have failed to show any sustained increase in the metabolic rate of 5 normal dogs under parathormone administration. In fact, if any significance is to be attached to the differences between the rates for the control period and for those under parathormone administration, there was a tendency to decrease the rate, with some tendency in two dogs to greater fluctuations in the rate as determined. However, it is doubtful if these differences are of significance.

The results of viosterol administration (4) were sometimes obtained in dogs in which there was no significant elevation of blood calcium concentration and on the other hand extreme elevation of the concentration (24–30 mgm.) frequently occurred without any effect on metabolism. In this series of 5 dogs the blood calcium was always increased by parathormone. It appears then that the "calcinosis" action of viosterol bears no direct relation to its calorigenic effect. Since the results obtained in dogs with viosterol could not be reproduced with extremely large doses of parathormone, it must be concluded that the parathyroids are not directly involved in this particular phase of the action of viosterol.

These results confirm the conclusions of Dale, Marble and Marks (2) and of Harris (1) that large doses of viosterol do not function by stimulation of the parathyroids. A recent review by Bills (5) gives a more detailed discussion of the evidence on this point.

SUMMARY

Large doses of parathormone administered to normal dogs do not increase the basal metabolism as was noted following the administration of viosterol.

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THE HYPOGLYCEMIC PHASE OF THE DEXTROSE TOLERANCE CURVE[1]

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In a previous communication (1) it was shown that normal dextrose tolerance curves can be obtained in depancreatized dogs, which are receiving a constant intravenous injection of insulin plus dextrose just sufficient to maintain the blood sugar at a constant level. Hepatectomized dogs, however, receiving a constant injection of dextrose sufficient to maintain a normal blood sugar level, yield consistently "diabetic" tolerance curves. By these and correlative data it was shown that the pancreas is not essential to the metabolic reactions which determine the normal dextrose tolerance curve, while the liver is essential. It was concluded that in the presence of a sufficiency of insulin, but not necessarily an extra secretion from the pancreas, the normal liver, as one of its responses to administered dextrose, decreases the output of blood sugar, which it has been supplying from its own resources.

In two (out of eight) of the normal dextrose tolerance tests, obtained as above in depancreatized dogs, the final blood sugar values fell below the pre-test levels. Since these results seemed particularly significant of the homeostatic liver mechanism which we have postulated, the present experiments were designed to magnify this hypoglycemic phase.

METHODS. Our previous procedure (1) was modified so as to utilize a constant intravenous injection of sugar as the test of carbohydrate tolerance, in accordance with the work of Felsher and Woodyatt (2), Thalhimer, Raine, Perry and Buttles (3), Jordan (4), and others on normal dogs and humans.

Completely depancreatized dogs were given constant injections of insulin plus dextrose sufficient to maintain a constant normal blood sugar level. After a suitable initial control period, an additional constant injection of dextrose was superimposed for three hours. The injection of extra sugar was then stopped, while the original insulin plus dextrose was continued.

[1] Presented before the American Physiological Society, March, 1934.

[2] Aided by the Max Pam Fund for Metabolic Research.

Unanesthetized normal and diabetic dogs, trained to lie quietly on the table, were used for all these experiments. The constant injections and blood sampling were done by venous puncture with hypodermic needles. All food and insulin were withheld for 18 hours previous to these tests. The pancreatectomies were performed under Pento-Barbital Sodium (Abbot) anesthesia 24 hours prior to the experiment.

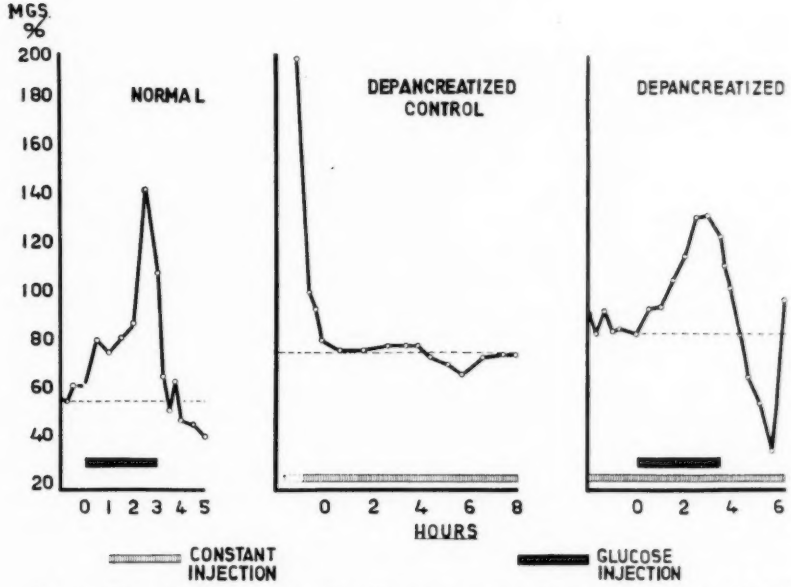

Fig. 1. The solid black line labelled *Glucose injection* refers to the injection of the test sugar. The cross-hatched line labelled *Constant injection* refers to the constant injections of insulin plus dextrose required to maintain a normal blood sugar level in the depancreatized dog.

Normal—Results of prolonged dextrose injection in normal dog.

Depancreatized Control—Maintenance of a normal blood sugar level in a depancreatized dog.

Depancreatized—Results of prolonged dextrose injection in a depancreatized dog with an established normal blood sugar level.

Results. Typical experiments are illustrated in the accompanying figure. Additional experiments (2 "normals," 2 "depancreatized controls" and 6 "depancreatized") show no significant difference from those presented, and are omitted to conserve space.

Normal. This curve illustrates the results of the constant intravenous injection of $\frac{1}{4}$ gram dextrose per kilogram per hour for three hours in a normal dog. It may be seen that the blood sugar level rose steadily but

began to decline before the cessation of the glucose administration. When the glucose was stopped, the blood sugar continued to fall for two hours. The final blood sugar level was 42 mgm. per cent as compared to the pre-injection level of about 60 mgm. per cent.

Depancreatized control. This curve shows the prolonged maintenance of a normal blood sugar level in a completely depancreatized dog by the constant injection of insulin plus dextrose. The arrow indicates the point at which one unit of insulin was administered subcutaneously in order to more quickly bring the blood sugar level down to the range at which it was desired to establish the constant level. Where the cross-hatching begins, the final adjustment of the proportion of constant insulin injection to the constant dextrose injection was made. The respective rates necessary for this particular dog were 0.07 unit insulin per kilogram per hour and 0.25 gram dextrose per kilogram per hour. It may be seen that for as long as these injections were continued (8 hours) the blood sugar level remained constant within an extreme variation of 14 mgm. per cent.

Depancreatized. This curve illustrates the results of the constant intravenous injection of $\frac{1}{4}$ gram dextrose per kilogram per hour for three hours in a completely depancreatized dog, in which a constant blood sugar level had been established as in the "depancreatized control." The constant blood sugar level was maintained for two hours before the superimposed injection of the test glucose was started. The rates of injection necessary to maintain the normal blood sugar level in this animal were 0.08 unit insulin per kilogram per hour and 0.25 gram dextrose per kilogram per hour respectively. In this animal, as in the normal, the blood sugar began to decline before the cessation of the administration of the extra sugar. When the latter was stopped the blood sugar level continued to fall for two hours, then returned to the previously established level. The low point of the hypoglycemic phase was 36 mgm. per cent as compared to the pre-test level of about 86 mgm. per cent.

SUMMARY AND DISCUSSION. These results confirm the results and conclusions presented in our previous communication (1). It has been shown that, under the conditions of our experiments, the completely depancreatized dog evidences at least as great a hypoglycemic reaction following the cessation of prolonged sugar administration as does the normal dog. Both organisms also show an increase in tolerance, as judged by the fall in the blood sugar level, while the test glucose is still being injected. These phenomena cannot therefore be ascribed, as Jordan (4) and others have done, to an increased mobilization of insulin from the pancreas in response to the administered sugar. Like the normal dextrose tolerance curve (1), they can be accounted for by the decrease in the supply of sugar by the liver to the blood, in response to the influx of exogenous sugar. This homeostatic mechanism, which has been previously discussed (1), occurs

in the presence of a sufficiency of circulating insulin, but does not require an extra secretion from the pancreas. The period of hypoglycemia, following the cessation of dextrose injection, therefore corresponds to the time which elapses before the liver is able to accelerate its rate of supply of blood sugar to a point sufficient to maintain the original normal blood sugar level.

Our work supports the views of Jordan (4) as to the arbitrary nature of the figures for sugar tolerance or utilization limit reported by earlier workers. As this author puts it "A truer measure of tolerance should be based, it would seem, on the rate at which glucose can be utilized under optimal conditions such as only exist when the resources of the body for disposing of glucose have been previously completely mobilized." It was during these "optimal conditions" that he found administered insulin to be inert. It seems probable from our results that his conditions were optimal and insulin administration was ineffective, not because "the tissues of his animals already contained an optimal concentration of insulin," but because the liver, at that time, had already made its maximum possible adjustment to the incoming sugar.

CONCLUSIONS

1. The hypoglycemic reaction following the cessation of prolonged sugar administration does not depend upon an increased mobilization of insulin from the pancreas.

2. This phenomenon represents another aspect of the homeostatic liver mechanism which has been postulated to explain the normal dextrose tolerance curve.

3. The bearing of this work on the conception of "glucose tolerance" has been briefly indicated.

We wish to express our appreciation to Mr. Fred E. Overthun and Miss Bernice Huddlestun for technical assistance, and to the Department of Chemistry for aid with the chemical determinations.

We are indebted to Eli Lilly & Company for the insulin used in these experiments.

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THE INFLUENCE OF FASTING AND ENVIRONMENTAL TEMPERATURE UPON THE ATROPHY OF DENERVATED SKELETAL MUSCLE

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This investigation was undertaken in order to determine whether or not the atrophy of skeletal muscle following denervation would be influenced by the state of nutrition of the animal. Experiments were carried out concerning the effects of glycine administration, changes in external temperature and fasting on the rate of denervation atrophy of the gastrocnemius muscle of the rat. In some of the experiments, determinations of the water, total nitrogen and creatine contents of normal and atrophic muscles were made. Attempts have been made to determine whether or not skeletal muscle in the absence of a nerve supply would regain any of the ponderable weight lost during a period of fasting prior to the denervation.

EXPERIMENTAL METHODS. Full-grown rats were used as experimental animals. The gastrocnemius of one side was denervated by removing a section of the sciatic nerve at the level of the trochanter. The corresponding muscle of the opposite limb served as a control.

A group of ten operated rats were given daily intraperitoneal injections of 0.2 gram of glycine. Five of the animals were killed one week and the remaining ones two weeks after denervation and the weights of the control and denervated muscles determined.

Twenty animals were used in experiments concerning the effect of environmental temperature upon the rate of denervation atrophy. Ten of the animals were placed in a refrigerator kept at a temperature of 16 to 20°C. The other ten were housed in a well ventilated incubator kept at a temperature of 31°C. Five animals from each group were sacrificed after one week and the remainder at the end of two weeks and the weights of control and denervated muscles compared.

A series of 56 experiments was carried out in a study of the effect of fasting upon the atrophy of denervation. The animals, after operation, were placed in individual metabolism cages and deprived of all food except water for periods of 3, 5, 7 or 14 days. Some of the fasted animals showed a tendency to eat their own denervated limb. This was no doubt initiated

by their licking at trophic skin lesions. It was found that this difficulty could be overcome by painting the denervated limb with a saturated solution of picric acid. Animals with extensive skin lesions were discarded. At the end of a designated period the animals were weighed and killed. The gastrocnemius muscles of the control and denervated limbs were carefully dissected, weighed and subjected to chemical analysis. An additional series of 10 animals was fasted for periods of 10 to 12 days. During this time the animals lost approximately one-third of their initial body weight. The sciatic nerve was then sectioned and the animals were placed on their usual diet, ad libitum, for a period of two weeks. At the end of this time the animals were sacrificed and the muscles treated as in

TABLE 1

The effect of environmental temperature and of glycine administration upon the rate of atrophy of denervated muscle

EXPERIMENTAL CONDITION	PER CENT OF DRY WEIGHT LOST FROM MUSCLE	
	7 days after denervation	14 days after denervation
Control animals	24.9	52.8
Standard deviation	5.8	4.0
Animals kept at 16–19°C	28.7	50.1
Standard deviation	2.8	1.6
Animals kept at 31°C	27.9	53.5
Standard deviation	5.4	3.2
Animals receiving glycine	25.9	51.1
Standard deviation	1.5	3.8

the above groups. A group of 57 animals from the same stock served as controls for the above experiments.

RESULTS AND DISCUSSION. It is evident from the results (table 1) that the administration of glycine had no effect upon the rate of atrophy of denervated muscle. Likewise this amino acid did not prevent the usual fall in the creatine content of the atrophic muscle. These observations are included because of the recent interest in a possible therapeutic rôle of glycine in certain types of clinical myopathies.

The results (table 1) indicate that environmental temperatures well above and below those ordinarily encountered in laboratories do not influence the rate of weight loss due to denervation atrophy. This finding stands in contrast to the well known effect of low environmental temperatures upon the weight loss in fasting. The food consumed per unit of

body weight at the lower temperature was 70 per cent greater than at the higher temperature. These findings indicate that variations in room temperature and the amount of food consumed, provided that the diet is adequate to maintain body weight, are without appreciable effect upon rate of atrophy of denervated muscle.

In order to determine the loss of ponderable weight from muscles during periods of concomitant fasting and denervation atrophy, it is necessary to know the original weights of such muscles. For this purpose we have

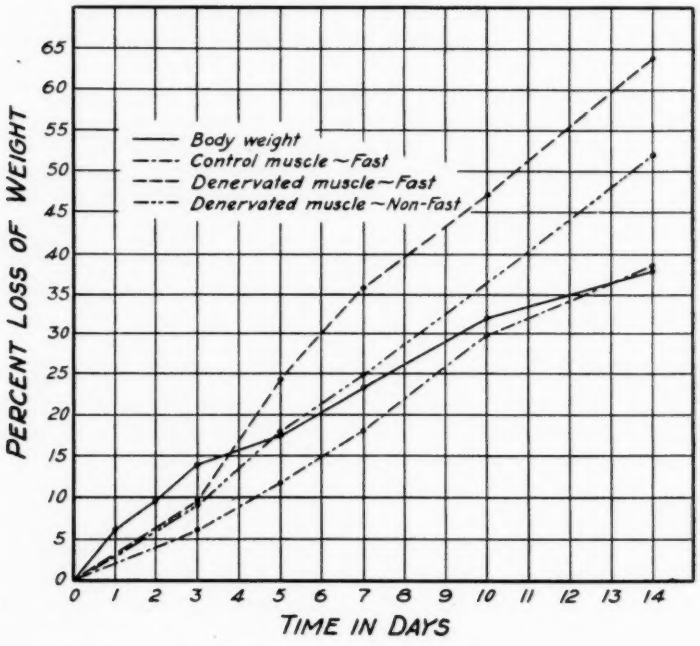

Fig. 1

determined the ratio of the weight of the gastrocnemius muscle to body weight in a series of 165 normal well fed adult rats. It was found that the mean weight of the gastrocnemius muscle was equal to 0.64 per cent of body weight (S.D. ± 0.0528. P.E.M. ± 0.0028). This value has been used in order to calculate the values referred to as original muscle weights in this report.

The rate of weight loss from the body and the different tissues during fasting depends upon a number of conditions such as age, nutritional state, degree of activity and external temperature. In figure 1 are plotted the

data relative to the loss of body weight occurring in a series of 100 animals subjected to periods of fasting ranging in duration from one to fourteen days. Likewise, the loss of weight in the gastrocnemius muscle has been calculated by comparing the actual weight found at any designated period with the calculated original weight. No attempts were made to extend the periods of fasting beyond two weeks because, at the end of this time, most of the animals were near the critical level of weight loss.

Although only slight changes were noted in the water content of the fasted and denervated muscles, the amounts of weight loss are expressed in terms of dry weight (table 2). The finding of approximately the same

TABLE 2

A summary of the average values concerning the effects of fasting and denervation upon muscle weight and composition

EXPERIMENTAL CONDITION	PER CENT OF DRY WEIGHT LOSS			WATER		CREATINE		TOTAL NITROGEN	
	Calculated*		Relative**	Per cent		Milligrams per 100 grams fresh muscle		Per cent (wet basis)	
	Control	Atrophy	Atrophy	Control	Atrophy	Control	Atrophy	Control	Atrophy
7 day atrophy non-fast			24.9	75.66	76.34	460	423	3.66	3.62
7 day atrophy and fast	18.0	35.8	21.4	75.97	76.84	457	426	3.53	3.50
14 day atrophy non-fast			50.2	76.43	77.31	469	349	3.67	3.50
14 day atrophy and fast	38.6	63.8	40.9	75.43	76.77	487	338	3.49	3.51
12 day fast plus 14 day atrophy and refeeding	21.3	62.9	52.8	76.16	76.92				

* Based upon calculated original muscle weights.

** Based upon actual weights of matched pairs.

nitrogen content in normal, fasted and atrophic muscles indicated that an equivalent loss of protein material had occurred in both conditions.

The results of these experiments indicate that skeletal muscle undergoes considerably more weight loss during periods of concomitant fasting and denervation than during similar periods of time with either condition alone. However, the total weight loss is less than the sum of the losses encountered when these conditions occurred independent of each other. When the weight of a denervated muscle is compared with that of its control in fasting animals, it is noted that the per cent of weight loss is less than that found in experiments on well fed animals. This may indicate either that fasting per se decreases the rate of weight loss due to atrophy or that muscles undergoing denervation atrophy contribute less material to the fasting quota than do normal control muscles. In view of these

findings it is evident that care must be exercised in drawing conclusions from experiments concerning the rate of denervation atrophy in which the control muscle loses appreciable weight.

The data (table 2) show that if an animal is fasted until it loses slightly more than one-third of its muscle and body weights and is then denervated and refed for a period of two weeks, the weight lost by such denervated muscles is as great as that found during a similar period of concomitant fasting and denervation. This finding indicates that a muscle in the absence of its nerve supply is unable to regain the ponderable weight lost in a period of fasting prior to the denervation. It would appear as if this phase of anabolism in muscle is lost or greatly suppressed following denervation and that such muscles have lost the property of protoplasmic synthesis.

In some respects the losses in weight encountered by the gastrocnemius muscle of the rat due to fasting and to denervation atrophy are similar. In both conditions the muscle undergoes a rapid weight loss without an appreciable change in the concentration of water and total nitrogen. Both conditions are accompanied by a lowering in the concentration of muscle glycogen.

In many respects, however, these two types of weight loss appear to be quite different. The weight loss due to fasting is accelerated by a decrease in environmental temperature; whereas that occurring in denervation atrophy is quite independent of environmental temperature. It has been found that skeletal muscle can lose as much as one-fifth of its weight due to fasting and yet retain a normal capacity to perform work (Leese, Hines and Jordan, 1932). Studies made on muscles undergoing atrophy of denervation point to an impaired functional capacity of such tissues (Caro, 1926). Fasting leads to a loss of muscle weight without a decrease in the concentration of functionally significant compounds such as phosphorus and creatine. In fact, Chanutin and Silvette (1928) have observed an increased concentration of the latter in the muscle of the rat during fasting. This stands in contrast to the decreased concentration of these substances noted in rats' skeletal muscle during denervation atrophy (Hines and Knowlton, 1933).

It may be postulated that skeletal muscle possesses at least structural, functional and storage components. Acute inanition is able to deprive the muscle of its storage fraction without appreciably reducing that concerned with function. Denervation appears to be followed by a decrease in both functional and storage components. It is possible that an equilibrium may exist between these components of muscle somewhat similar to that shown by Holman, Mahoney and Whipple (1934) to exist for proteins between blood and tissues. It is suggested that the so-called trophic effect of nerve on skeletal muscle is concerned primarily with the

development and maintenance of the functional components concerned with the thermodynamics of contractility. After denervation the muscle suffers a loss of these important substances and of the anabolic processes required to bring about protoplasmic synthesis.

SUMMARY

This report is concerned with the influence of glycine administration, external temperature and fasting on the denervation atrophy of skeletal muscle. The studies were made on the gastrocnemius muscle of the rat. They include observations on the rate of weight loss and the concentration of water, total nitrogen and creatine in control and denervated muscle.

It was found that the administration of 0.2 gram of glycine per day did not influence the rate of muscle atrophy.

No difference was noted between the rate of atrophy of muscles in animals kept at an environmental temperature of 16 to 19°C. and those kept at 31°C.

The denervated muscles of fasting animals lost weight at a faster rate than that due either to fasting or denervation alone; but the total loss was less than the sum of the losses encountered by control muscle during fasting and by denervated muscle under non-fasting conditions.

It was found that denervated muscle was unable to regain the ponderable weight lost in a period of fasting prior to the denervation.

It is postulated that fasting causes primarily a loss of storage fractions from the muscle rather than material concerned directly with contractility. Denervated muscle appears to lose the fractions concerned with storage and function and suffers a loss in the property of anabolic synthesis as well.

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THE EFFECT OF POSTURE ON THE MINUTE VOLUME OF THE HEART[1]

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The effect of posture on the minute volume of the heart has been the subject of considerable experimentation. The methods of investigation have been many and the results of the investigations have not been concordant but, until recently at least, the balance of evidence has been in favor of the view that the output of the heart increases progressively in changing from the erect to the sitting and to the recumbent positions (6, 1, 2, 5, 8). Grollman (3) has challenged this conclusion, however, and has presented evidence in favor of the idea that the heart output remains constant in subjects at rest in various positions. He summarizes briefly the experimentation concerned with the effect of posture on the heart output in his paper (3).

Grollman (3, 7) made a study of the time necessary for thorough mixing of gases in a lung-bag system, such as is employed in determining the heart output, when the subject was in various positions. The research demonstrated that while 15 seconds of rebreathing was an adequate time for mixing to occur in the sitting or standing positions, the time would be lengthened to 18 seconds when the subject was lying down. This necessity he attributed to the decrease in the vital capacity of the subject when recumbent. This experimentor (3) then reinvestigated the subject of the minute volume of the heart when subjects were in different positions. He employed the nitrous oxide method described by Marshall and Grollman (7) and came to the conclusion that when gas samples were collected at 18 and 23 seconds, respectively, thereby allowing time for complete mixing of the gases in the lung-bag system, the results showed that the heart output was unaltered when the subjects remained quietly in different postures. He therefore attributed the results of earlier workers, who had shown heart output increases in the standing compared with the recumbent position, to withdrawal of gas samples before adequate mixing of gases had occurred in their rebreathing systems.

During the present year the Grollman acetylene method (4) has been

[1] The expense of this investigation has been met by a grant from the Charles Himrod Denison Fund.

employed in this laboratory in connection with certain metabolic studies. As an incidental part of our work, the heart output has been determined on several subjects both in standing and in reclining positions, and our results substantiate the older point of view attacked by Grollman. The subjects were familiar with the rebreathing procedure, having submitted to this experimentation several times, and the gas samples were collected in accordance with Grollman's instructions; i.e., at the 18th and 23rd seconds, respectively. Seven respiratory cycles were completed by the time the first sample was taken.

The subjects were healthy male students with the exception of E. S. who is a middle-aged man in good health. Ordinarily the subjects came into the experimental room after having sat through a 50-minute class lecture; they remained seated at rest for at least five minutes before any determinations were made. In making the first determination for heart

TABLE 1

Lying down vs. standing

SUBJECT	ARTERIO-VENOUS O_2 DIFFERENCE		CARDIAC OUTPUT		STROKE VOLUME		O_2 CONSUMPTION	
	Standing 1′	Lying down 15′ or more	Standing 1′	Lying down 15′ or more	Standing 1′	Lying down 15′ or more	Standing 1′	Lying down 15′ or more
	ml. per liter		*liters*		*ml.*		*ml.*	
E. S.	78	45	3.7	5.0	46	64	285	255
W. R.	76	60	3.6	4.4	62	99	270	260
S. R.	69	59	3.8	4.2	54	75	260	240
M. T.	84	51	5.1	6.5	85	108	425	230
M. B.	79	54	3.7	5.2	42	72	290	280

output the subject stood up, put the mouthpiece in place, then stood quietly for one minute before beginning the rebreathing procedure. Following this the subject reclined for 15 minutes or longer and upon being connected with the lung-bag system again rebreathed as before.

Table 1 gives the result of a series of experiments. Without exception the subjects showed an increase in the output of the heart after assuming a recumbent position for 15 minutes as compared with the output while standing.

The first two columns of table 1 show larger arterio-venous oxygen differences of our subjects when standing than when reclining. This increased withdrawal of oxygen from the blood is regarded as a compensatory reaction which counteracts the effect of a diminished circulation in the upright position.

When acetylene is rebreathed in a concentration of about 10 per cent for 20 seconds or so most subjects experience a slight dizziness which

vanishes upon breathing pure air. All our subjects experienced a much profounder sense of giddiness when rebreathing the acetylene lying down than when standing even though the time of rebreathing and the concentration of acetylene were the same in both positions. This increased sense of dizziness is indicative of a greater absorption of acetylene and is quite in accord with the increased output of the heart in recumbency.

In order to determine the effects of long, quiet standing on the output of the heart, the same subjects were used. The first determination was made after the subject had stood for one minute as described above. After a short rest the subject then stood *absolutely quiet*—but not at rigid attention—for at least 15 minutes. The second rebreathing process was accomplished some time between the 15th and 20th minute. None of the men of this group complained of dizziness upon standing for that period of time and there was no external evidence of circulatory embarrassment. Apparently these men compensated for the effect of gravity on the circulation

TABLE 2

SUBJECT	ARTERIO-VENOUS O_2 DIFFERENCE		CARDIAC OUTPUT		STROKE VOLUME		O_2 CONSUMPTION	
	Standing 1′	Standing 15+′	Standing 1′	Standing 15+′	Standing 1′	Standing 15+′	Standing 1′	Standing 15+′
	ml. per liter		*liters*		*ml.*		*ml.*	
E. S.	68	70	3.8	4.0	44	43	260	280
W. R.	75	84	3.7	3.5	61	57	275	290
S. R.	75	88	3.7	3.3	45	39	280	290
M. T.	86	90	4.8	4.4	68	54	415	395
M. B.	91	93	3.1	3.0	37	35	280	280

during long standing much better than the female subjects used by Turner (9, 10) who inferred an insufficient heart output in subjects whose pulse pressures fell below 20 mm. Hg during prolonged standing. Pulse pressure below 20 mm. in a standing subject is almost always accompanied by dizziness. Blood pressure determinations were taken repeatedly on two of our subjects (W. R. and S. R.) during prolonged standing but their pulse pressures never decreased to 20 mm. Hg and generally were in the vicinity of 30 mm. Hg. Table 2 indicates a tendency for the minute volume of the heart to decrease during prolonged standing, but the change is small and in most cases within the limits of experimental error. Table 2 presents averages of two determinations on each subject, all of whom had served at least twice before in determinations for heart output.

Two students were discovered whose pulse pressures dropped below 20 mm. Hg on long standing. These men consented to act as our experimental subjects for heart output studies.

A. B. served twice; in each experiment he stood longer than 15 minutes. His pulse pressure was 12 at the end of the first experiment and 16 at the end of the second, but he felt no discomfort except for pains in his legs after the first few minutes of standing. His heart output showed a distinct decrease in both experiments.

Subject A. B.

ARTERIO-VENOUS O_2 DIFFERENCE		CARDIAC OUTPUT		STROKE VOLUME		O_2 CONSUMPTION	
Standing 1′	Standing 15+′	Standing 1′	Standing 15+′	Standing 1′	Standing 15+′	Standing 1′	Standing 15+′
ml. per liter		*liters*		*ml.*		*ml.*	
65	78	5.4	3.9	55	40	350	300
62	81	5.2	3.6	46	33	320	295

F. H. becomes dizzy on standing quietly for some time. In our first experiment with him he complained of discomfort at the end of eight minutes and thus the second rebreathing procedure was accomplished at that time. The heart output, however, was found to be precisely the same after 8 minutes' standing as after 1 minute (i.e., 4.3 liters). The stroke volume, however, was reduced from 60 ml. to 42 ml. due to a marked acceleration of the heart after standing 8 minutes. Three days later the same subject repeated the experiment and was able to stand 15 minutes, at which time, however, he was decidedly dizzy and his skin pale. The heart output was found to have decreased from 4.0 liters to 2.8 liters and the stroke volume from 57 ml. to 28 ml. During a third experiment F. H. fainted at the 15th minute just before the second rebreathing procedure. He was revived and after sitting a minute again resumed a quiet standing position for 3 minutes. A heart output determination made at the end of the third minute showed no decrease in volume compared with the original output after standing one minute.

Presumably both A. B. and F. H. have less perfect vasomotor control than the first group studied with a consequent pooling of blood in the extremities and splanchnic area which thus seriously impairs the venous return to the heart.

SUMMARY

We conclude from results obtained on seven subjects that:

1. The minute volume of the heart is increased in the recumbent as compared with the erect posture.

2. Ordinarily on prolonged *quiet* standing the output of the heart either remains unchanged or decreases slightly in subjects exhibiting no distress as a result of the long standing.

3. The heart output is decreased in subjects who, on long quiet standing, display poor circulatory compensation in the erect position. A pulse pressure below 20 mm. of mercury indicates a falling cardiac output.

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DIABETIC HYPERPYREXIA[1]

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Crandall in 1899 was the first to recognize a hyperpyrexia of purely anhydremic origin. He described in undernourished children attacks of fever which disappeared when water was given. Bakwin (1922) also demonstrated that the inanition fever of newborns is associated with an increased concentration of the blood. With the administration of fluids both the concentration and temperature returned to normal. Polyuria (Balcar, Sansum and Woodyatt, 1922) resulting from the injection of hypertonic glucose solution produced anhydremia and hyperpyrexia, phenomena which disappeared on the injection of an isotonic solution.

Similarly the polyuria of diabetes is a potential cause of anhydremia and hyperpyrexia. Lande (1933) called attention to the clinical importance of diabetic hyperpyrexia unassociated with any infectious process. This communication describes a study of the syndrome characterized by hyperpyrexia in depancreatized dogs.

METHOD. The observations were made on twenty-four dogs rendered completely diabetic by aseptic removal of the pancreas. The animals were maintained on a diet of meat and sugar to which pancreatin (Lilly) was added. Insulin was injected twice daily.[2] The dogs were usually in good condition for two weeks after the operation before experimental study.

It is well known that the production of polyuria in diabetic animals merely requires removal of insulin. In these experiments both insulin and water were withheld in order to produce dehydration rapidly.

During the development and regression of the anhydremia blood samples were drawn and analyzed for CO_2 and O_2 content and capacity, glucose, lactic acid and pH, using the methods of Van Slyke and Neill (1924), Hagedorn and Jensen (1923), Friedemann, Cotonio and Shaffer (1927), and

[1] The results have been described in part in an address by H. E. Himwich at the Annual Graduate Fortnight of the New York Academy of Medicine, 1933. (Bull. N. Y. Acad. Med. **10:** 16, 1934.) Aided by a grant from the Research Funds of the Yale University School of Medicine.

[2] The insulin was generously supplied by Eli Lilly & Company through the courtesy of H. W. Rhodehamel, Director of Research.

DuBois (1932) respectively. The specific gravity and osmotic pressure of the serum were determined by the methods of Barbour and Hamilton (1926) and Hill (1930) and total base and chloride by the techniques of Stadie and Ross (1925) and Eisenman (1929). With the aid of thermocouples the temperature of various parts of the body was studied. Finally changes of the heart were recorded by means of an electrocardiograph.

RESULTS. Table 1 presents results typical of an experiment obtained on dog 5. Columns 2, 3, 4 and 5 illustrate the concomitant rise and fall of the rectal temperature with the variation in the concentration of the blood. The specific gravity and osmotic pressure of the serum, as well

TABLE 1

Changes during development and regression of diabetic hyperpyrexia

1	2	3	4	5	6	7	8	9
Water intake	Temp.	Sp. gr.	Osm. pr.	O_2 capt. vol. %	Lact. acid mgm.%	pH	Alk. res. vol. %	Acetone mgm. %
Ad lib	99.5°	1.0245	0.894	20.04	25	7.38	40.13	Not done
None for 30 hours	104.2°	1.0315	1.149	24.83	45	7.25	32.40	2.5
700 cc. 3 hours	102.2°	1.0254	1.099	20.69	70	7.18	32.32	0
1000 cc. 10 hours	100.4°	1.0246	1.016	19.58	16	7.29	37.92	4.2
Ad lib. 26 hours	99.5°	1.0234	0.876	19.74	17	7.35	37.99	Not done

TABLE 2

Effect of the ingestion of water on diabetic hyperpyrexia (no insulin was injected)

WATER INTAKE	TEMP.	BLOOD SUGAR mgm. %	SP. GRAVITY	LACTIC ACID mgm. %
Ad lib	100.6°	258	1.0248	22
None for 24 hours	104.5°	490	1.0275	54
1.5 liters, 3 hours	99.3°	520	1.0227	42
Ad lib., 24 hours	100.0°	360	1.0213	25

as the oxygen capacity of the blood, increased when water, insulin and glucose were withheld. On the subsequent administration of glucose in saline and injection of insulin the temperature fell as the specific gravity, osmotic pressure, and oxygen capacity diminished. Columns 2, 6, 7, 8 and 9 show that the concentration of lactic acid of the blood also increases and falls with the temperature, while the pH and alkaline reserve of the blood vary inversely with the concentration of lactic acid. The changes of acetone substances are of no great significance. In other experiments (table 2) water was administered orally as the sole therapeutic agent to reduce the hyperpyrexia. Despite the omission of insulin and glucose the course of the defervescence was unaltered as is evidenced by the regression

of the changes of temperature, specific gravity of the serum and lactic acid of the blood.

TABLE 3

Acid base balance of serum (milliequivalents)

TEMP.	BASE	CHLORIDE	$NaHCO_3$	LACTIC ACID
100.4	133	104	19	1.8
104.5	152	121	14.1	6.1
99.1	133	104	21.0	1.4

TABLE 4

Lactic acid of urine (mgm. per 24 hours)

	BISULFITE BINDING SUBSTANCES		LACTIC ACID EXCESS
	During fever	After fever	During fever
Dog no. 15	1131	180	1151
Dog no. 16	1929	114	1885

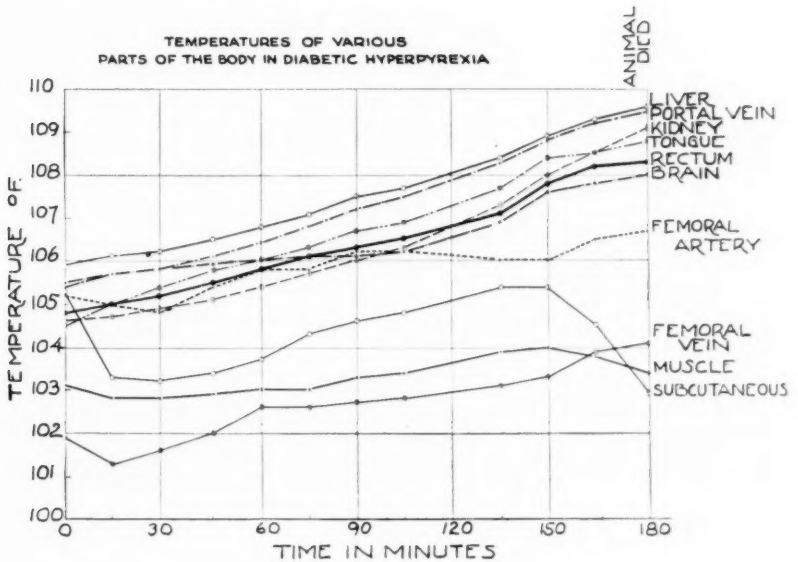

Fig. 1

The effect of the accumulation of lactic acid on the acid base equilibrium of the serum was studied. In a characteristic experiment the bicarbonate

diminished despite the increased concentration of the serum, a concentration which caused the rise of total base and chlorides (table 3).

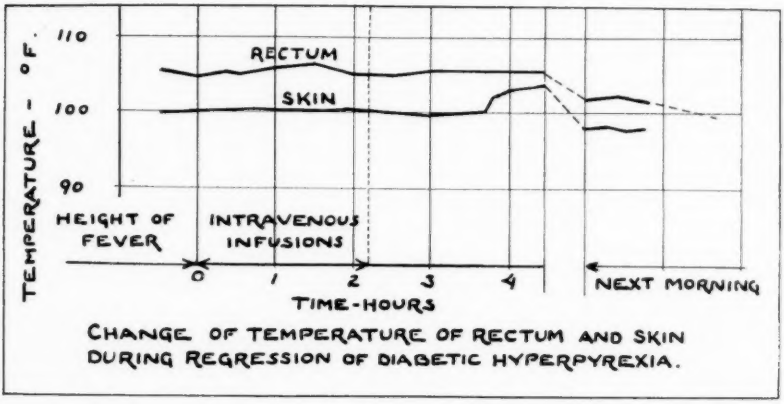

Fig. 2

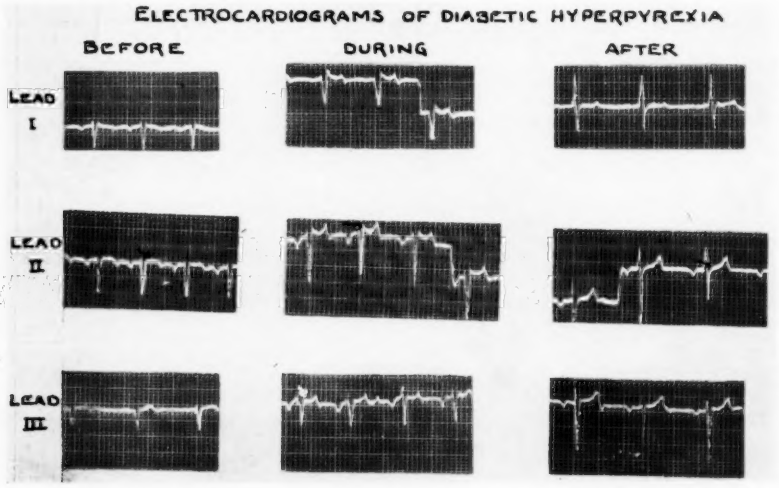

Fig. 3

The high level of the lactic acid in the blood permits that substance to leave the body through the kidneys. An analysis of the urine during fever reveals an increase of bisulfite-binding substances (table 4).

Next the temperature of 3 dogs was recorded during the development of the fever. When the rectal temperature had risen above 100° Fahren-

heit the animals were anesthetized with sodium amytal and thermocouples inserted in various organs. Readings were taken every fifteen minutes until thermic death occurred. Figure 1 shows the variations in temperature of the various organs during diabetic hyperpyrexia. Of the internal organs the liver is the warmest, while the periphery, including muscular and subcutaneous tissue, is relatively cold.

Temperature changes during defervescence were also studied. Dog 4 (fig. 2) was anesthetized with sodium amytal after his temperature had mounted to 105.5°F. One thermocouple was inserted in the rectum and the other placed on the shaved skin of the thorax. After the initial readings had been made 1.0 liter of isotonic saline (38°C.) was administered intravenously. For some time there was no marked change. One and one-half hour after the infusion, however, the skin temperature rose almost to 104.0°F., while the rectal temperature continued at the same high level for a longer period. The next morning both the rectal and skin temperatures had retrogressed toward the normal values.

The heart is also affected by the anhydremic fever for the S.T. segment of the electrocardiogram is raised above the isoelectric line (fig. 3). However, within twenty-four hours after the ingestion of water the S.T. segment resumes its normal position on the isoelectric line.

Discussion. The temperature of the arterial blood (fig. 1) is lower than that of the rectum (Wright and Johnson, 1933) since arterial temperature is influenced to a great extent by that of the periphery of the body. The arterial fluid is a mixture of the warm blood coming from the internal organs and the cooler blood coming from the periphery of the body after passage through the lungs and heart. The temperature of the arterial blood is therefore the resultant of the temperatures of the venous blood coming from the various parts of the body. The thermal differences between the internal organs are not great and the temperatures of all the internal organs vary together although the liver is warmer than the kidney, brain, portal vein, tongue and rectum (fig. 1). The lower rectal temperature reveals that it, like that of the arterial fluid, is influenced by the thermic condition of the periphery of the body. Nevertheless the close agreement between the temperature of the rectum and that of the other internal organs in our experiments makes it a good indicator of the thermal condition of the organs of the body.

Barbour and Gilman (1934) have noted increased specific gravity and osmotic pressure in fevers produced by a variety of pyretic agents, and have described their significance. The results of our investigation demonstrate the correlation between the rectal temperature and the concentration of the blood. The high specific gravity, osmotic pressure and oxygen capacity are all evidences of a diminished blood volume in diabetic fever. With an inadequate blood volume and the imperative requirements of the

vital viscera the blood supply to the skin, the chief mediator of heat loss, is decreased as is evidenced by its diminished temperature. Furthermore, the blood that does enter the cutaneous vessels is less effective in the loss of heat since an increase of osmotic pressure causes a marked decrease of insensible perspiration (Gilman and Barbour, 1933). Thus the elimination of heat cannot cope with its production and fever results. The importance of the regulation of heat loss is seen not only in the production of fever, but also during defervescence, since not until the skin temperature rises, does the rectal fall. There is therefore an inverse ratio between skin and rectal temperatures, the skin temperature decreases as the rectal approaches a maximum, while in recovery from fever the skin temperature rises before the rectal is able to fall (fig. 2).

The fact that in the experiment on dog 3 the skin temperature did not change for some time after the intravenous infusion indicates that the arterioles of the skin were shut at first and did not permit blood to enter the cutaneous vessels. Fremont-Smith and his co-workers (1929) have demonstrated the closure of the skin capillaries during the development of fever. Such action is added evidence for the point of view that the skin was kept in a relatively bloodless condition because of reflex closure of its smaller vessels, which relaxed only some time after the fluid content of the body had been increased. The same mechanism probably obtains in the musculature of the extremities since these muscles, like the skin, are also relatively cold during the development of the fever (fig. 1). Marriott (1920) observed that blood flow in the extremities of anhydremic babies was reduced to one-fifth to one-tenth that of normal infants. As a result of the relative anaerobiosis lactic acid accumulates in the muscles and pours out in the blood. The data of Hartmann and Darrow (1926) reveal an increased concentration of lactic acid in the blood of some of their patients with diabetes. In the experiment on dog 5 (table 1) the lactic acid of the blood continued to rise from 45 to 70 mgm. per cent even though the blood volume had increased and the temperature had begun to fall, 102.2°F., due to the oral administration of saline and glucose and the injection of insulin. Probably with the improved blood flow the lactic acid that had accumulated in the muscles was washed out and the pH of the blood thereby diminished from 7.25 to 7.18. Bicarbonate did not fall with this further decrease of pH since the base of the blood was raised by the ingestion of the salt.

Hewlett, Barnett and Lewis (1926) demonstrated that the renal threshold for lactic acid lies between 30 to 40 mgm. per cent. The high level of blood lactic acid is therefore reflected in the urine (table 4). The urinary lactic acid as indicated by the bisulfite binding substances is increased.

The specific gravity, osmotic pressure and viscosity of the serum are changed by the anhydremia. The greater specific gravity of the serum is

probably caused by the increased concentration of serum protein. The increased osmotic pressure of the serum is due chiefly to the greater concentration of total base and chloride, the decrease of bicarbonate being balanced by the increase of lactate. The diminution of serum volume, demonstrated by increased osmotic pressure and specific gravity is also evident in the raised oxygen capacity, an indication of a greater number of red blood corpuscles per unit volume of blood. This combination of inspissation of the serum and relative increase of the formed constituents of the blood makes for its greater viscosity. The increased concentration of the serum indicates a similar change of the tissues since the extra- and intracellular fluids are probably in osmotic equilibrium. Certainly the tissues of our animals appeared highly desiccated.

Despite the diminished blood flow of the skin and muscles the oxygen supply of the heart is also inadequate as indicated by the change of position of the S.T. segment (fig. 3). In the present experiments the increased viscosity of the blood is one of the factors tending to diminish blood flow. In addition it is possible that the inspissation of the tissues affects directly the enzymes of oxidation. Whatever the exact mechanism may be the electrocardiogram reveals changes occurring during anoxemia of the cardiac musculature. Nahum and Hoff (1934) state that the S.T. segment leaves the isoelectric line whenever the oxygen uptake of the heart is insufficient. McCulloch's (1920) electrocardiograms of anhydremic children reveal a rise of the S.T. segment, exactly the change noted in the anhydremic dog (fig. 3). Thus the entire syndrome of diabetic hyperpyrexia results from anhydremia with inspissation of the blood as presented schematically below:

Anhydremia with inspissation of the blood	decrease in blood supply to skin → hyperpyrexia
	decrease in blood supply to muscle → lactic acid
	changes in E.K.G. similar to those of insufficient oxygen supply to heart.

Further evidence for this conception of diabetic hyperpyrexia is afforded by the fact that the administration of fluids restores all the changes characteristic of this condition to their original status and at the same time saves the life of the animal (table 2). A sharp differentiation must be made between the therapeutic effects of insulin and fluids. The injection of insulin is effective in diminishing blood sugar and acetone substances, but only the administration of fluids can increase the blood volume evident in the diminution of specific gravity, osmotic pressure and oxygen capacity, and thus permit the reëstablishment of blood flow through the cutaneous,

skeletal and cardiac vessels adequate to reduce the high temperature, diminish lactic acid and thus raise bicarbonate and pH and finally to restore the S.T. segment of the electrocardiogram to its normal position.

The syndrome described by Lande (1933) probably has a pathogenesis similar to the hyperpyrexia of our dogs. However, in severe diabetes hypothermia is a more frequent occurrence than is hyperthermia. One must inquire what determines the direction of the thermal development in this condition. In hyperpyretic dogs with anhydremia the blood was inspissated while patients with diabetes who may develop a fall in temperature have a low blood volume characterized by dilution of the blood (Chang, Harrop and Schaub, 1928; Peters, Kydd and Eisenman, 1933). It would therefore appear that with anhydremia and inspissation of the blood hyperpyrexia supervenes, while with low blood volume and dilution of the body fluids, hypopyrexia and even shock may ensue. Depancreatized dogs receiving no insulin but drinking water do not develop hyperpyrexia and exhibit a progressive dilution of the blood. Dilution of the electrolytes and proteins of the serum permits fluid to leave the capillaries. The resulting depletion of the blood volume leads to a fall of blood pressure, and the development of subnormal temperature and shock. When the concentration of the blood is increased, however, the blood volume is better maintained chiefly because of the greater osmotic and oncotic pressures. These with the greater viscosity, tend to maintain blood pressure and prevent shock.

In diabetes the kidney eliminates increased amounts of sugar, electrolytes and acetone substances although the last are not marked in the canine species. Whether the concentration of the blood decreases or increases will depend upon the relative volume of water simultaneously lost from the body. In many instances the factor determining the concentration of the blood is a temporal one. During a protracted process the body is given the opportunity to accommodate itself to the anhydremia and ingested water is retained despite the decreased concentration of electrolytes and serum proteins. Whereas in the acute condition such a relative retention of water can not take place. From this it is evident that the chief requirement of the body during diabetic hyperpyrexia is water, and the administration of water and not insulin and glucose is of primary importance for the alleviation of this condition.

SUMMARY

A syndrome in dogs closely resembling the diabetic hyperpyrexia of human patients is described, with certain signs which may be sought in human patients. The basis of the syndrome is the polyuria, the cause of the anhydremia. The inspissation of the blood prevents the complete loss of water from the capillaries and thus aids in the maintenance of blood

volume and blood pressure, while the decreased volume is the direct etiological factor in the production of the syndrome. Because of the lack of blood in the skin heat-loss is diminished and fever ensues, a phenomenon which can be reversed by the reëntrance of blood in the cutaneous vessels after administration of water. The accumulation of lactic acid in the blood and the resulting fall of pH and alkaline reserve as well as the increased urinary lactic acid are caused by an inadequate blood supply of the voluntary muscles. In the heart the same anoxemic condition develops as is evidenced by changes of the electrocardiogram. Thus the functional pathology indicates an insufficient volume of blood due to loss of water and therefore the effective means of treatment consists in increasing the water content of the body.

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THE RELATION OF THE SECRETION OF MUCUS TO THE ACIDITY OF THE GASTRIC JUICE

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Many workers have tried to explain the nature and causes of the normal variations which have been observed in the acidity of the gastric juice. The three theories that have been advanced to explain these variations differ in the point of view as to the importance of neutralization by gastric mucus in causing them.

The first theory is that of Pavlov (1) who stated that "the gastric juice as it flows from the glands possesses a constant acidity." He attributed the apparent variations in acidity to neutralization by alkaline mucus. However, he did not exclude the possibility of a relationship between acidity and the rate of secretion.

The second hypothesis is that of Rosemann (2) who objected to Pavlov's theory on the ground that all of the mucus would be washed out of the stomach during the earlier part of the experiment and that consequently neutralization by mucus could not account for the decreased acidity observed in the latter part of the experimental period. He believed that the gastric cells secreted chloride ion at a constant concentration, and that the amount of hydrochloric acid formed from the chloride depended upon the secretory energy of the cells.

The third view is that of Maclean and his associates (3) who concluded that gastric acidity is not normally controlled by neutralization. They believed that "the presence of a certain concentration of acid in the stomach inhibits secretion of acid with the result that a neutral fluid containing chloride is secreted which, by dilution, reduces the acidity of the gastric contents."

Recently, Bolton and Goodhart (4), Hollander (5), and Wilhelmj, Neigus, and Hill (6), by means of experiments on animals, have furnished strong evidence in favor of Pavlov's theory. In the studies described in this paper on adult human subjects, evidence is presented to show the important rôle of mucus in determining the degree of acidity of the gastric contents. A method of calculating the concentration of hydrochloric acid as secreted by the parietal cells is also presented.

METHODS AND PROCEDURE. No food or drink was given to the subjects between the evening meal and the morning of the test. Early in the morning the fasting contents were removed by means of a Rehfuss tube, after which 0.5 mgm. of histamine hydrochloride was injected subcutaneously. Subsequent samples were collected at 20-minute intervals over a

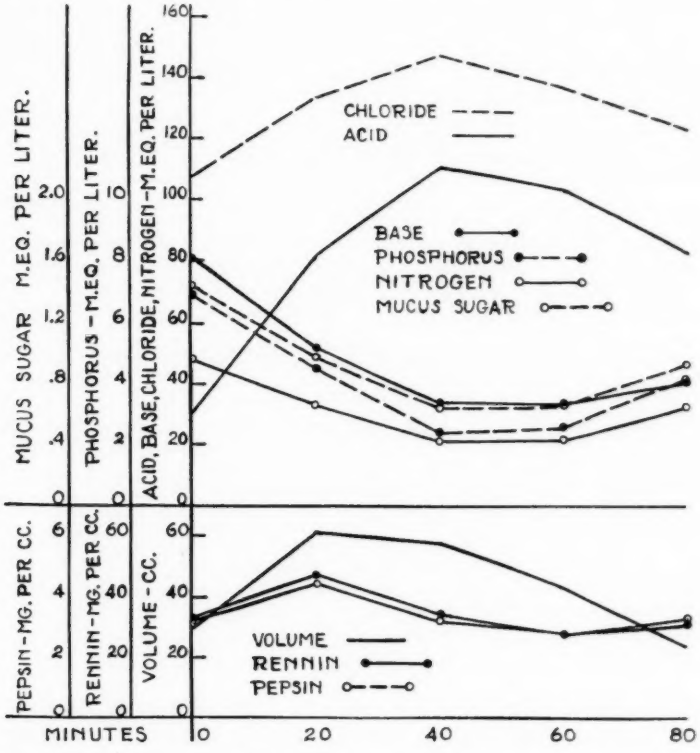

Fig. 1. The average of the gastric analyses on 10 human subjects after histamine stimulation.

period of 80 minutes, great care being taken to remove as completely as possible all of the gastric contents at each withdrawal.

Each specimen of gastric juice was measured in a graduated cylinder and was filtered through paper before using for analysis. The titratable acid was determined on 1 cc. of gastric juice, titrating with N/100 sodium hydroxide to pH 7.0 using phenol red as an indicator. Chlorides were determined by the method of Van Slyke and Sendroy (7).

The mucus in the gastric contents was estimated by hydrolyzing the

gastric juice with 3 per cent hydrochloric acid, neutralizing with sodium hydroxide, and determining the reducing sugars by means of the revised copper method of Folin (8). The results were expressed in milli-equivalents of glucose per liter.

Since the total base, phosphorus, and nitrogen have been considered by various authors as components of the mucous secretion, these constituents were determined. The total base was determined by the method of Wright and Allison (9). Fiske and Subbarow's (10) method was used for the determination of phosphorus. Nitrogen was determined by the manometric method of Van Slyke (11).

The enzyme secretions, being protein in nature, would contribute to the nitrogen content. Therefore, pepsin was determined by the Koch and

TABLE 1

The averages of the concentrations of titratable acid, chlorides, total base, phosphorus, nitrogen, pepsin, rennin, and the calculated hydrochloric acid in the gastric contents of 10 human subjects after histamine stimulation

SPECIMEN	VOL.	ACID	CHLORIDES	TOTAL BASE	PHOSPHORUS	NITROGEN	MUCOUS SUGAR*	CALC. TOTAL BASE	PEPSIN†	RENNIN‡	CALC. CONC. OF HCl
	cc.	*Milli-equivalents per liter*							*mgm. per cc.*	*mgm. per cc.*	*m.-eq. per liter*
Fasting	30	30	107	81	6.9	46	1.44	77	3.2	33	120
20′	61	82	134	52	4.5	33	0.98	52	4.4	47	156
40′	57	111	148	34	2.4	21	0.64	37	3.2	34	161
60′	43	104	138	34	2.6	22	0.66	34	2.8	28	152
80′	24	84	124	41	4.2	33	0.94	40	3.3	31	149

* Calculated as m.-eq. of glucose.
† Calculated as mgm. of 1:4000 U. S. P. pepsin.
‡ Calculated as mgm. of U. S. P. rennin.

McMeekin (12, 13) method and expressed in milligrams of 1:4000 U. S. P. pepsin per cubic centimeter, and the rennin was determined by a modification of the Michaelis (13) method and expressed in milligrams of U. S. P. rennin per cubic centimeter.

Results. The results of the fractional gastric analyses after histamine stimulation on 10 human subjects are shown in table 1. The concentration of the chloride in these studies is not as constant as that found in experiments on pouch dogs. However, there is a much greater increase in titratable acid than chloride. The increase in acid is accompanied by a corresponding decrease in total base, nitrogen, phosphorus, and complex sugar (mucoitin sulfuric acid). The relationship of these substances to one another is shown clearly in figure 1. The curves for total base, nitrogen, phosphorus, and complex sugar show a distinct parallelism. There-

fore, these substances are all probably components of the same secretion (the mucous secretion), especially since the reducing sugar after hydrolysis is one of these components. Of course, part of the nitrogen is due to the enzyme secretion, although, as will be shown later, the enzyme secretion contributes only a small part of the nitrogen. Martin (14) has also shown that a part of the nitrogen is due to non-protein nitrogenous substances. Furthermore, the variation in the total base is due chiefly to changes in the sodium content, since Gamble and McIver (15) and Austin and Gammon (16) have shown that the potassium content of the gastric juice remains fairly constant regardless of the acidity. The latter authors have also shown a parallelism between the nitrogen and sodium content of the gastric juice. The pepsin and rennin values, which closely parallel each other, are not related to the titratable acid; the greatest enzyme concentra-

TABLE 2

Composite of 10 fractional gastric analyses after histamine stimulation, showing the rate of secretion

SPECIMEN	VOL.	ACID	CHLORIDES	TOTAL BASE	PHOSPHORUS	NITROGEN	MUCOUS SUGAR	PEPSIN	CALC. HCl SECRETED BY CELLS
	cc.	*milli-equivalents per specimen*						*mgm. per specimen*	*m.-eq. per specimen*
Fasting	30	0.90	3.21	2.42	0.208	1.93	0.043	95	1.96
20′	61	4.99	8.16	3.16	0.272	2.82	0.060	267	6.76
40′	57	6.33	8.43	1.94	0.135	1.68	0.036	183	7.76
60′	43	4.48	5.92	1.44	0.110	1.34	0.028	122	5.41
80′	24	2.01	2.98	0.98	0.101	0.65	0.022	79	2.57

Total pepsin = 746 mgm.
Calculated as crystalline pepsin = 53 mgm.
Nitrogen due to pepsin = 7.95 mgm. or 0.57 m.-eq.
Total nitrogen = 8.42 m.-eq.

tion usually precedes the peak of the acid concentration. The data in table 1 will be used later for calculating the concentration of the hydrochloric acid in the parietal secretion.

In table 2 are shown the total amounts of the determined constituents of the gastric juice secreted in each 20-minute period. The average amount of pepsin secreted during the whole test period for these 10 analyses was 746 mgm. of 1:4000 U. S. P. pepsin. Since Northrop's (17) pepsin is equivalent to 1:56000 U. S. P. pepsin (or 14 times more active), the average amount of pepsin secreted in this series of experiments is equivalent to 53 mgm. of crystalline pepsin. Crystalline pepsin contains 15 per cent Kjeldahl nitrogen; therefore, only 7.95 mgm., or 0.57 m.-eq., of nitrogen are due to the pepsin secretion. Since the total nitrogen found during this period was 8.42 m.-eq., the nitrogen due to pepsin does not influence the curve of nitrogen secretion to any great extent.

Another important fact brought out in this table is that after histamine stimulation there is an increase in the amounts of total base, nitrogen, phosphorus, and complex sugar in the 20-minute period. Although these components decrease with each succeeding sample, the total amounts of these constituents are reduced to only one-third of the amount that is present at the peak of their secretion. This means that throughout the experimental period mucus is present and in large enough quantities to have a definite neutralizing effect, especially at the beginning and at the end of the experiment when the flow of parietal secretion is smaller. These findings are contrary to Rosemann's views and are in accord with those of Webster (18). In studies on a dog with a Heidenhain pouch Webster measured the amount of mucus by centrifuging in graduated tubes or by weighing the mucus collected by filtering the juice through alundum filters.

The peak of the pepsin secretion also occurs in the same period as the mucous secretion; however, there is a much greater output of enzyme than of mucus, since, as shown in figure 1, the mucous components decrease in concentration in this period whereas the enzyme concentration is increased. The peak of the titratable acid and chloride secretion occurs in the 40-minute period, again showing the lack of correlation between the acid and enzyme secretory mechanisms.

On the basis of the data presented, the conclusion has been drawn that to histamine stimulation there are at least three distinct secretory responses in the stomach: the parietal secretion of hydrochloric acid, the secretion of enzymes, and the secretion of mucus. The increase in mucus in the gastric contents, however, may be due to its being flushed into the stomach by the force of the acid and enzyme secretions.

Therefore, believing that the mucus by its neutralizing effect on the parietal secretion plays an important rôle in determining the amount of titratable acid found in the gastric contents, an attempt has been made to calculate the concentration at which hydrochloric acid is secreted by the parietal cells of the gastric mucosa.

METHOD OF CALCULATION. The chief difficulties in any calculation on gastric juice obtained from human subjects are duodenal and salivary contamination. Duodenal contamination can be practically eliminated by using for analysis only those samples which are found to be free from bile. However, the saliva presents a more difficult problem. This difficulty has been compensated for in the following manner. Gamble and McIver's (15) data show that in the gastric juice from cats with fundic pouches the ratio of sodium to phosphorus when calculated in milli-equivalents is approximately 10 to 1, regardless of the acidity. The data of Clark and Shell (19) show that in human saliva the ratio of sodium to phosphorus is 0.62 to 1. That is, the ratio of the sodium to the phosphorus in the mucous secretion is considerably greater than this ratio in the saliva.

Therefore, knowing this ratio in a mixture of mucus and saliva, the percentage of each of these components can be determined from a graph made by plotting the figures in table 3 which records ratios of sodium to phosphorus given by hypothetical mixtures of mucus and saliva. The composition of saliva used in obtaining these ratios and for later calculations was that of Clark and Shell (19): sodium 9 m.-eq. per liter, phosphorus 14.5 m.-eq. per liter, and chlorides 14.1 m.-eq. per liter. The actual composition of the mucous secretion is very difficult to determine by chemical analysis because one cannot be sure that the acid-producing cells are at rest. The analysis of the gastric contents of patients with achylia gastrica cannot give this information, since the mucous membranes of such patients cannot be considered to be normal, even if salivary contamination is eliminated. Hollander (5) (1932), by means of data obtained from pouch dogs, has calculated that the chloride content of the mucous secretion is equivalent to 100 m.-eq. per liter. Wilhelmj, Neigus, and Hill (6) have confirmed this figure with an average value of 103 m.-eq. Bolton and Goodhart (4) reported a minimum chloride value of 99 m.-eq.

TABLE 3

Ratio of base to phosphorus in a mixture of mucus and saliva

Parts of mucus	10	9	8	7	6	5	4	3	2	1	0
Parts of saliva	0	1	2	3	4	5	6	7	8	9	10
Ratio of base to phosphorus	10	9.14	8.24	7.33	6.42	5.50	4.56	3.59	2.62	1.63	0.62

for the mucous secretion in experiments on cats. Gamble and McIver (15) and Austin and Gammon (16) have reported higher chloride values for their determinations on the mucus from fundic and pyloric pouches. The latter authors stated, however, that accurate analysis was difficult due to the highly viscous nature of the mucus.

Recent work has shown that most glandular secretions are approximately isotonic with the blood (5, 15, 20). Therefore, considering the mucous secretion to be such a solution and the ratio of sodium to potassium to be 10 to 1, as stated before, the values for mucus used in the calculations in this paper are: sodium 157 m.-eq. per liter, chloride 100 m.-eq. per liter, and phosphorus 15.7 m.-eq. per liter.

The method of calculating the concentration of the hydrochloric acid secreted by the parietal cells is as follows. The data of Gamble and McIver (15) show that the sum of the amounts of potassium and calcium in the gastric juice is fairly constant (approximately 16 m.-eq.). Therefore the sodium content of the gastric juice can be calculated with reasonable accuracy even in the presence of salivary contamination, for the sum of the

potassium and calcium components of the saliva is 17.8 m.-eq. (19). The data of the 40-minute sample of table 1 will be used for an example of the calculation. This sample contains: chloride 148 m.-eq. per liter, total base 34 m.-eq. per liter, and phosphorus 2.4 m.-eq. per liter.

	$34 - 16 =$	18.0	m.-eq. of Na due to mucus and saliva
The ratio of Na to P $= \frac{18}{2.4}$	$=$	7.5	
From the data in table 3 the value will give 72 parts of mucus and 28 parts of saliva			
Therefore, in a liter of such a mixture.....	$0.72 \times 157 =$	113.0	m.-eq. of Na due to mucus
	$0.28 \times 9 =$	2.5	m.-eq. of Na due to saliva
or		115.5	m.-eq. of Na due to mixture
Since the sample contained 18 m.-eq. of Na due to mucus and saliva.....			
	$\frac{18}{115.5} =$	0.156	liter of mucus and saliva per liter of gastric juice
Therefore.....	$1.000 - 0.156 =$	0.844	liter of HCl per liter of gastric juice
Since the ratio of mucus to saliva is 72 to 28.....	$0.72 \times 0.156 =$	0.112	liter per liter of juice due to mucus
	$0.28 \times 0.156 =$	0.044	liter per liter of juice due to saliva
Mucus contains 100 m.-eq. of Cl_2 per liter			
Therefore.....	$0.112 \times 100 =$	11.2	m.-eq. of Cl_2 per liter due to mucus
Saliva contains 14.1 m.-eq. of Cl_2 per liter			
Therefore.....	$0.044 \times 14.1 =$	0.62	m.-eq. of Cl_2 per liter due to saliva
or a total of Cl_2 not due to HCl of.....	$11.2 + 0.62 =$	11.82	m.-eq.
Therefore.....	$148 - 11.8 =$	136.2	m.-eq. of Cl_2 due to HCl
Therefore the concentration of HCl.....	$= \frac{136.2}{0.844} =$	161.0	m.-eq. per liter

The calculated concentration of the hydrochloric acid, as secreted by the parietal cells, for each period is shown in the last column of table 1. The concentration of hydrochloric acid at the height of the secretion is isotonic with the blood. Due to the complex nature of the gastric secretory response these values are only approximate. The actual determination of

the sodium content would increase the accuracy of the calculations. Also, data obtained from cats and dogs have been freely transposed. However, these values are in good agreement with the data obtained from studies on dogs by Hollander (168 m.-eq.) and Wilhelmj, Neigus, and Hill (163 m.-eq.), considering especially the small amounts of histamine used for stimulation in these studies compared to those on dogs.

The relation of the calculated hydrochloric acid concentration to its rate of secretion is shown in figure 2. These curves show that the concentration of acid may be affected to a slight degree by the rate of secretion. In all experiments of this type it is difficult to calculate the diluting effect of the enzyme secretions. As shown in table 1, the concentration of pepsin and rennin is increased in the 80-minute sample and may play a part in reducing the calculated hydrochloric acid value in this period.

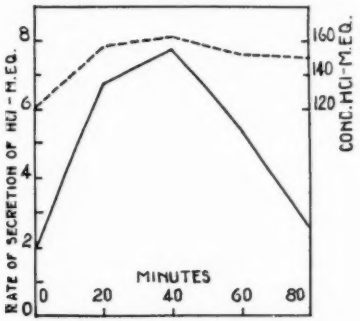

Fig. 2. The relation between the rate of secretion and the concentration of the hydrochloric acid secreted by the parietal cells, after histamine stimulation.
(Solid line—rate of secretion; broken line—concentration.)

SUMMARY AND CONCLUSIONS

The data in this paper show that mucus is present in the gastric contents throughout the experimental period in sufficient quantities to account for the main variations in the titratable acid of the gastric juice by neutralizing and diluting the hydrochloric acid secreted by the parietal cells. The ratio of 10 to 1 for sodium to phosphorus in the mucous secretion compared to the ratio of 0.62 to 1 for the sodium to phosphorus in the saliva was used to estimate the extent of salivary contamination in the gastric contents. This correction made it possible to calculate the concentration of the hydrochloric acid secreted by the parietal cells. The concentration of hydrochloric acid was found to be 161 m.-eq. per liter, which is approximately isotonic with the blood.

These studies on human gastric contents, in a like manner to those on experimental animals, favor the hypothesis advanced by Pavlov to explain the normal variations in the acidity of the gastric juice.

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THE VARIATION OF ERYTHROCYTES, HEMOGLOBIN AND PACKED CELL VOLUME IN IMMEDIATELY CONSECUTIVE SAMPLES OF VENOUS BLOOD

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Exact serial studies on the hemoglobin and cells of blood in normal individuals for the purpose of identifying and establishing the magnitude of various physiological fluctuations have necessitated careful quantitative estimation of the total error inherent in all phases of the method.

While some investigators have made short statistical studies on the limits of error in various routine methods employed in blood counting and in determination of hemoglobin and packed cell volume, apparently the question of possible deviations due to the variable distribution of cells in the blood stream has not been examined quantitatively.

Smith, Arnold and Whipple (1921) have called attention to the unequal distribution of cells in the axis and periphery of the stream in smaller vessels. Microscopic examination shows that most of the cells are in the center of the stream, where they flow, to use the expression of Fahraeus (1929), "within a plasma tube." According to the calculations of Smith and his collaborators, this "still space" may constitute one-eighth the diameter or 44 per cent of the cross sectional area of the vessel.

If the observations made microscopically on smaller vessels are applicable to the larger veins, a needle inserted in various positions might be expected to withdraw samples of blood having quite different erythrocyte and hemoglobin values. The present study was made to ascertain the magnitude of any such differences. Immediately consecutive samples of blood, withdrawn as rapidly as possible by venous puncture, have been studied in a statistical examination of this type of variation.

METHODS. Ten healthy male students between the ages of 18 and 25 were used as subjects. A 20-gauge needle was inserted into the median cubital vein and 10 cc. syringes were applied in rapid succession until ten 6 cc. samples had been withdrawn. The manipulations were sufficient to vary the position of the needle in the vein considerably from one sample to the next. No tourniquet was used.

After withdrawal, each sample was thoroughly mixed with sufficient dry heparin to prevent clotting. The total withdrawal time for each subject

averaged about four minutes, ranging from two to eight minutes in individual cases.

All counts and readings were made by the author. A 1 cc. portion of the heparinized blood was used for cell counts and hemoglobin determinations. Erythrocytes were counted after dilution with Hayem's solution in U. S. Standard Trenner pipettes and three minutes' mixing in a mechanical shaker. Standardized Levy-Hausser counting chambers with improved Neubauer ruling were used in counting. Two counts agreeing within 100,000 cells were made from separate pipettes for each sample.

Hemoglobin was estimated by the Newcomer method in a colorimeter standardized by the oxygen capacity method. Six readings of the colorimeter scale were averaged for each diluted sample. Readings were made

TABLE 1

SUBJECT NO.	NUMBER OF SAMPLES	ERYTHROCYTE COUNT				QUANTITY OF HEMOGLOBIN				VOLUME OF PACKED CELLS			
		Mean	Std. dev.	Coeff. of var.	Max. dev.	Mean	Std. dev.	Coeff. of var.	Max. dev.	Mean	Std. dev.	Coeff. of var.	Max. dev.
		million				*gm./100 cc.*				*cc./100 cc.*			
1	10	5.25	0.157	2.99	0.26	15.97	0.202	1.26	0.34	50.7	1.17	2.31	2.6
2	8	4.94	0.082	1.67	0.15	15.64	0.143	0.92	0.26	48.2	1.32	2.74	2.3
3	10	5.11	0.142	2.78	0.25	14.49	0.369	2.54	0.77	47.4	0.59	1.25	0.9
4	10	5.46	0.100	1.83	0.22	17.01	0.357	1.67	0.65	51.6	1.01	1.95	1.7
5	10	5.31	0.063	1.19	0.10	16.63	0.419	2.52	0.73	49.7	1.08	2.17	1.8
6	10	5.49	0.080	1.45	0.19	18.02	0.179	1.00	0.36	52.5	1.09	2.08	2.5
7	10	5.04	0.086	1.70	0.21	14.58	0.329	2.26	0.45	46.3	1.00	2.16	2.0
8	10	5.06	0.086	1.69	0.16	15.68	0.164	1.05	0.22	48.3	0.96	1.99	1.7
9	9	5.06	0.127	2.52	0.26	14.78	0.213	1.44	0.36	49.0	1.07	2.19	1.9
10	10	5.84	0.079	1.35	0.12	17.99	0.284	1.58	0.71	55.7	0.73	1.30	1.2
Mean.....		5.26	0.100	1.92		16.08	0.266	1.62		49.9	1.00	2.01	

by north daylight after the acid hematin solution had stood for at least one hour.

Packed cell volume was ascertained by placing 5 cc. of the heparinized blood in a 12 cc. centrifuge tube and packing to constant volume at 3600 r.p.m. in a type 1SB International centrifuge (working radius, 9 cm.). The samples were rotated for one hour, removed for reading, and recentrifuged for 20 minutes. Packing was usually complete at the first reading.

Discussion. The observations are given in condensed form in table 1, only the mean values of each series being shown, together with the standard deviation, coefficient of variation and the maximum deviation from the mean for each set of consecutive samples.

In table 2 are shown the means of the corresponding corpuscular constants for each series, calculated from the data of table 1 according to the method used by Wintrobe (1932).

In no case is the maximum deviation in any one of the ten series greater than four times the probable error of the observations making up the series in which it occurs. Even these maximum variations, therefore, are of no greater magnitude than those which one might expect to encounter due to chance in random sampling. This is true for erythrocytes, hemoglobin and volume of packed cells, as well as for corpuscular volume, corpuscular hemoglobin and corpuscular hemoglobin concentration.

Erythrocyte counts. Various investigators have attempted to ascertain the error of the methods used in red cell counts. Smith's (1931) review cites a number of these, indicating the limit of error estimated by each one. In most cases the coefficient of variation is less than 3 per cent.

TABLE 2

SUBJECT NO.	NUMBER OF SAMPLES	CORPUSCULAR VOLUME				CORPUSCULAR HEMOGLOBIN				CORPUSCULAR HB CONC.			
		Mean	Std. dev.	Coeff. of var.	Max. dev.	Mean	Std. dev.	Coeff. of var.	Max. dev.	Mean	Std. dev.	Coeff. of var.	Max. dev.
		cubic micra				*micromicrogm*				*per cent*			
1	10	97.4	3.21	3.29	4.7	31.6	0.50	1.58	0.9	31.9	1.16	3.64	2.4
2	8	96.4	3.67	3.81	5.6	30.4	0.97	3.18	1.7	31.5	0.74	2.34	1.4
3	10	92.9	2.48	2.67	4.4	28.4	1.32	4.65	2.1	30.3	0.79	2.61	1.6
4	10	94.5	2.09	2.21	3.9	31.2	1.10	3.52	2.2	32.9	1.22	3.72	2.3
5	10	93.5	1.54	1.64	4.1	31.3	0.84	2.69	1.9	33.3	1.32	3.96	2.4
6	10	95.7	2.47	2.58	4.7	32.9	0.61	1.84	1.1	34.3	0.73	2.13	1.6
7	10	92.0	2.07	2.25	4.6	29.0	0.99	3.41	2.1	31.5	1.08	3.42	2.0
8	10	95.4	2.20	2.31	3.4	31.1	0.67	2.15	1.3	32.5	0.59	1.82	1.5
9	9	96.8	1.39	1.44	2.6	29.2	0.96	3.30	1.6	30.2	0.86	2.86	1.6
10	10	95.4	1.19	1.25	2.9	30.8	0.64	2.08	1.8	32.3	0.39	1.22	0.9
Mean.....		95.0	2.23	2.35		30.6	0.86	2.84		32.1	0.89	2.77	

Smith's (1931) technicians checked their accuracy by counting 20 drops from the same pipette. This procedure, of course, gives a measure only of the errors appearing in manipulations subsequent to the filling of the pipette. It does not include the errors due to that operation, although filling the pipette is probably the greatest single source of error in the entire procedure of counting. This objection Smith overcame in part by counting from two pipettes until the difference of the means was insignificantly small, according to its probable error. Smith concludes that a coefficient of variation of less than 3 per cent should be attained.

Wintrobe (1930) made a number of consecutive dilutions and counts from the same sample of blood, calculating the probable error for the series. The coefficient of variation calculated from the typical series of dilutions which he gives is 2.12 per cent. This coefficient includes variations due to all manipulations subsequent to withdrawal of the blood, but does not

indicate how great the variations due to the method of sampling may be. Smith (1932) later made further tests by taking 12 successive samples of blood from the same subject, using, however, capillary blood from the finger tip. The coefficient of variation by this method was 2.8 per cent.

The coefficient of variation for erythrocyte counts in the present study averages 1.92 per cent. In only three of the ten series was the coefficient greater than 2 per cent. Thus, it is possible to make counts consistently with a variation approximating 2 per cent, including variations due to sampling from the vein.

Hemoglobin. In estimating hemoglobin with the Bausch and Lomb Newcomer hemoglobinometer, Osgood (1926) found errors as great as 12 per cent. Wintrobe (1930) concluded that the total possible error by the Newcomer method is 10 to 12 per cent. Smith (1931) tested the accuracy of the method by making 20 readings from the same pipette, deriving a coefficient of variation less than 1 per cent. No significant difference was found between readings from two pipettes filled from the same drop of blood.

In the present investigation, the mean variation in hemoglobin is 1.62 per cent, exceeding 2 per cent in only three of the ten series.

Packed cell volume. Wintrobe (1930) estimated the error of his method for obtaining packed cell volume (3 mm. bore hematocrit tubes) by making consecutive hematocrit determinations on the same sample of blood. He found variations between different samples of the same specimen of blood no greater than 0.5 per cent.

Similar studies by Walters and May (1935) on simultaneous determinations of packed cell volume in 12 cc. tubes, filled to 5 cc. with freshly-drawn heparinized dog's blood from the same container, show a standard deviation of 0.623 cc. Since the mean standard deviation found in the present study by an identical method is 1.00 cc., these wider variations appear to be due to the method of sampling.

Corpuscular constants. In order to ascertain the probable error of the methods used in deriving corpuscular constants, Wintrobe (1932) made 20 determinations on 4 samples of blood. The coefficients of variation calculated from these data were: mean corpuscular volume, 0.85 per cent; mean corpuscular hemoglobin, 1.13 per cent; mean corpuscular hemoglobin concentration, 0.98 per cent.

In table 2 are shown the mean coefficients of variation calculated in the present study. These figures indicate that variations in mean corpuscular volume, hemoglobin or hemoglobin concentration must be greater than 3 per cent in order to exceed the errors common to the methods employed in this investigation.

SUMMARY

1. The variations in erythrocyte count, quantity of hemoglobin and volume of packed cells, and in mean corpuscular volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration, found in series of immediately consecutive samples of venous blood drawn from normal men, are no greater than those to be expected in chance variations due to random sampling.

2. The coefficients of variation calculated in this study reflect deviations due to the method of sampling as well as the variations which occur in subsequent technical manipulations.

Laboratory facilities for this investigation were provided in Watkins Memorial Hospital through the courtesy of Dr. Ralph I. Canuteson, Director of Student Health Service at the University of Kansas. Messrs. Homer Hiebert and James May also gave valuable assistance.

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THE INTERRELATIONS OF VAGAL AND ACCELERATOR EFFECTS ON THE CARDIAC RATE

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Several studies have been made concerning the effects on the heart rate (h. r.) of simultaneous stimulation of the vagi and the accelerators (see Tigerstedt, 1921, for references). The conclusions drawn from the majority of these studies are qualitative. The only quantitative statement which we know is that of Hunt (1897), that the result of simultaneous stimulation of the two sets of nerve fibers is very nearly the arithmetical mean of the results of the isolated stimulations. The problem is of interest not only because of the reciprocal elevation and depression of the continuous tonic discharge in the two nerve supplies during reflex changes of h. r., but also because of its bearing on the mode of action of the autonomic nerves and on the interrelations of excitation and inhibition.

For these reasons the present quantitative study was undertaken.

METHOD. Cats were used, under dial anesthesia, which insures a stable basal h. r. if the temperature of the animal is maintained constant. The h. r. was recorded by means of a Marey tambour applied to the thoracic wall. The left vagus was stimulated in the neck. For cardio-accelerator stimulation the electrodes were applied to the internal branch of the right stellate ganglion, after severance of all the other connections of the ganglion. The right vagus was previously sectioned aseptically in the neck and time (about 8 days) was allowed for degeneration, to eliminate a possible perturbing spread when the accelerator was stimulated.

Maximal electric waves at variable frequencies were employed as stimuli. Each nerve was activated by a separate circuit so that independent variations of the frequencies could be obtained. The two stimulating circuits were a "multivibrator," delivering rectangular waves lasting about 1 σ, and a condenser set connected with a mechanical interruptor.

Artificial respiration was administered when the thorax was opened to operate on the stellate ganglia. The rectal temperature of the animals was maintained between 37 and 38°C. No precautions were taken to eliminate the adrenals, for dial abolishes any demonstrable reflex secretion of adrenine (unpublished observations).

Stimulation of the vagus for 10 seconds and the accelerator for 20 seconds

causes practically maximal changes of h. r.—i.e., a steady state is reached (see fig. 5). The vagus, therefore, was usually stimulated for 20 seconds, the accelerator for 30 seconds, and the h. r. during the last 10 seconds was recorded as the maximal response for the frequency employed.

In some animals high frequencies (usually above 8 per second) of stimulation of the vagus alone made the rate so slow that the ventricle escaped and beat at its own rhythm. This led to a break in the corresponding curves. Such observations were discarded.

RESULTS. A. *Effects of vagal stimulation with and without a simultaneous tonic accelerator discharge.* Cats under dial anesthesia have a per-

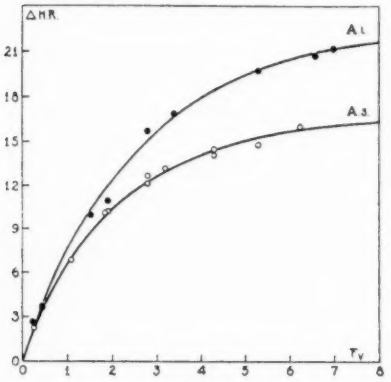

Fig. 1

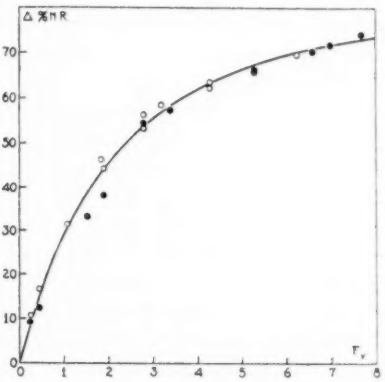

Fig. 2

Fig. 1. Vagi cut. Carotids ligated. Ordinates: maximal slowing of the h. r. in beats per 10 seconds. Abscissae: frequencies of maximal peripheral stimulation of the left vagus. The curve *A.i.* (dots) denotes the responses obtained with the accelerators intact; the curve *A.s.* (circles), with the accelerators severed.

Fig. 2. The data in figure 1 plotted as per cent of the basal rates. Ordinates: percental slowing. Abscissae: frequencies. Dots and circles as in figure 1. The basal rate with the accelerators intact was 30.2, ±1.3, and that after severance of the accelerators was 22, ±1 per 10 seconds.

sistent tonic accelerator discharge, especially marked if the vagi and depressors are sectioned and the carotids ligated. In such preparations the falls of blood pressure elicited by peripheral stimulation of the left vagus evoke a minimal or no change in this discharge because the main, if not all, circulatory proprioceptors have been inactivated. Under these circumstances, therefore, tonic accelerator impulses and stimulated vagal impulses are acting simultaneously, and the differences in the responses before and after severance of the accelerator supply give information concerning the rôle of each set of nerves when acting together.

Figure 1 illustrates these different responses. The left vagus was stimu-

lated for 20 seconds at various frequencies. The number of beats occurring during the last 10 seconds of stimulation was counted. The difference between this number and the basal h. r. per 10 seconds is plotted against the corresponding frequency. The curve *A. i.* represents the slowings obtained with the accelerators intact, the curve *A. s.* the responses after severance of the accelerators.

From these curves it would appear as if vagal stimulation were more effective when an accelerator discharge is present than when absent. The same data plotted as actual h. r. obtained (cf. fig. 11) would, on the contrary, give rise to the impression that the results of vagal stimulation are greater in the denervated heart. In figure 2, however, the slowings represented in figure 1 are expressed as per cent of the existing basal h. r. A single curve fits satisfactorily all the observations made. Similar results were found in all the preparations tested. We may conclude that the percental slowing elicited by a given stimulation of the vagus is the same whether a simultaneous accelerator tonic discharge is present or not.

B. *Simultaneous stimulation of the two sets of cardiac nerves at variable frequencies.* In order to eliminate the possibility of variations in the accelerator tone while the vagus was activated and in order to ascertain whether the percental slowing evoked by a given stimulation of the vagus is independent of the degree of accelerator activity in all the physiologic range, the following experiments were performed. The heart was disconnected from the central nervous system. A series of responses to stimulation of the left vagus alone at various frequencies was recorded. Similar series were then obtained during the persistent stimulation of the right accelerator at a given frequency, different for each series (cf. fig. 11). It was found that the responses thus elicited again yielded a single curve when plotted as per cent of the corresponding preëxisting rates. Figure 3 illustrates two series of responses to vagal stimulation, the one, V, without and the other, $V + A$, with simultaneous persistent activation of the accelerator at a frequency of 8 per second. Figure 4 shows the similarity of effects when plotted as per cent of the basal. A similar agreement of percental effects was found with all degrees of activation tried (up to 20 per second, which is probably the maximum that the systems involved may attain: see Rosenblueth, 1932). A similar agreement was likewise found when the accelerator was stimulated for 30 seconds at various frequencies while the vagus was persistently activated throughout a series. This latter procedure was not applied extensively because of the technical disadvantage which ensues from the slower recovery after accelerator stimulation as compared with the rapid return to basal after vagal excitation (see fig. 5).

C. *The time course of the responses to simultaneous stimulation of the vagus and the accelerators.* The results reported in the two previous sec-

tions show that the percental *maximal* effect of a given stimulus on one of the nerves is the same, no matter what degree of persistent simultaneous activation of the other nerve is applied. Are the percental effects *at any given moment* during simultaneous stimulation the same as those which would occur had one of the nerves been activated singly? The observations reported here furnish an answer to this question.

Figure 5 illustrates the time course of the responses to separate stimulation of the vagus, V, and of the accelerator, A, for 30 seconds, and to simultaneous excitation of the two nerves, $V + A$, with the same stimuli

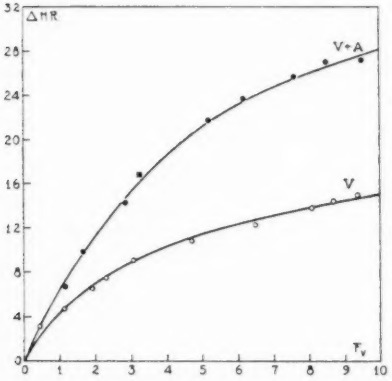

Fig. 3

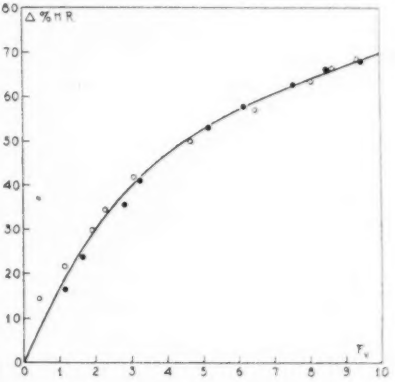

Fig. 4

Fig. 3. Right vagus sectioned previously, left vagus acutely. Both accelerators disconnected from the centers. Ordinates and abscissae as in figure 1. The curve V (circles) denotes the responses obtained on stimulation of the left vagus alone; the curve $V + A$ (dots), the responses to variable frequencies on the vagus and simultaneous persistent stimulation of the right accelerator with a frequency of 8 maximal shocks per second.

Fig. 4. The data in figure 3 plotted as per cent of the basal rates. Ordinates and abscissae as in figure 2. Dots and circles as in figure 3. The basal rate when the vagus alone was stimulated was 21.75 ± 0.15 per 10 seconds. The basal rate while the accelerator was persistently stimulated was 40.6 ± 0.6 per 10 seconds.

used for the separate activations. The slowing induced by the vagus alone is succeeded by a moderate acceleration, due probably to some accelerator fibers contained in the nerve (see Tigerstedt, *loc. cit.*). After simultaneous stimulation the slowing is promptly succeeded by marked acceleration, so that the h. r. rises to the values it would have had if the accelerator alone had been activated, and the two curves, A and $V + A$, thereafter almost coincide (cf. Baxt, 1875). A similarly close coincidence is observed on recovery from the slowing induced by stimulation of the vagus during the prolonged after-effects of previous excitation of the accelerator (fig. 6).

Figure 7 illustrates the effects of stimulating either the vagus, $A + V$ or the accelerator, $V + A$, while the other nerve was being continuously activated before (30 seconds or more) and throughout the observation. The frequencies employed on each nerve were the same as those applied for the responses recorded in figure 5. That the percental effects of each nerve on the rate imposed by the other are the same at any moment as would obtain if activated alone is shown in figure 8. The circles are the effects of the accelerator and vagus alone in figure 5, plotted as per cent of the basal rate of the denervated heart. The dots are the responses in

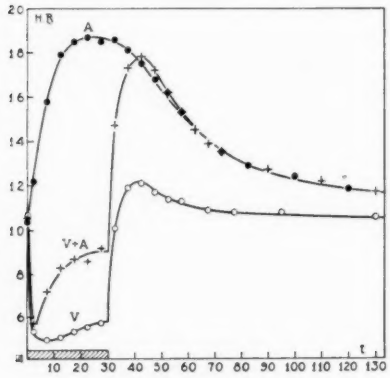

Fig. 5

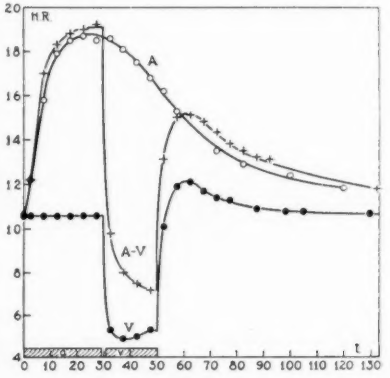

Fig. 6

Fig. 5. Ordinates: h. r. per 5 seconds. Abscissae: time in seconds. Stimulus for the 3 curves as marked. A (dots): right accelerator alone at 8 per second. V (circles): left vagus alone at 3.4 per second. $V + A$ (crosses): the two nerves together at the same frequencies as singly.

Fig. 6. Ordinates and abscissae as in figure 5. The right accelerator was stimulated during the period marked *a*, the vagus during that marked *v*. A (circles): accelerator alone. V (dots): vagus alone. $A - V$ (crosses): accelerator first, then vagus.

figure 7, plotted as per cent of the rates elicited by the persistent stimulation of either nerve. The percental effects are seen to be practically the same throughout the observations.

Discussion. The data presented (figs. 2, 4 and 8) show that a given stimulation of the decelerator nerves induces the same percental degree of slowing, independently of whether the accelerators are excited or not, other variables being kept constant (temperature, etc.); and *vice versa*, a given stimulation of the accelerators evokes a given percental increase of h. r. which is independent of decelerator excitation. Such being the case, the results of simultaneous stimulation of the two sets of nerves should in

general differ from the arithmetical mean of the separate effects. Figure 9 illustrates this difference. Curve V denotes the responses of a decentralized heart upon stimulation of the vagus alone at various frequencies. The accelerator was now persistently activated with a frequency of 8 per second, whereupon the h. r. went from 25 to 43.5 beats per 10 seconds. A series of responses to the vagus was then recorded, while stimulation of the accelerator was maintained. The broken line A. M. denotes the effects which would have been obtained if the responses to simultaneous activation were the arithmetical mean of the separate effects. The actual values

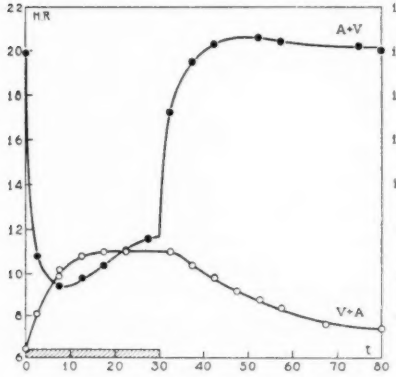

Fig. 7

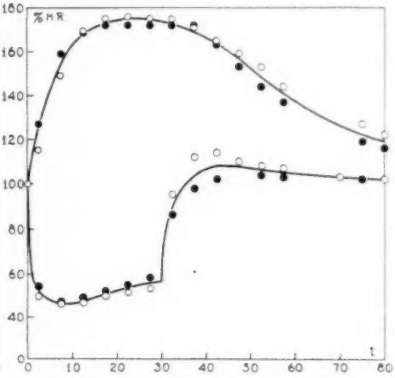

Fig. 8

Fig. 7. Ordinates and abscissae as in figure 5. $A + V$ (dots): right accelerator activated persistently at 8 per second before and throughout the observation; left vagus stimulated at 3.4 per second during the period indicated. $V + A$ (circles): vagus activated persistently at 3.4 per second before and throughout the observation; accelerator stimulated at 8 per second during the period indicated.

Fig. 8. Ordinates: per cent of the corresponding basal h. r. Abscissae: time in seconds. Circles: curves A and V of figure 5. Dots: curves $A + V$ and $V + A$ of figure 7. For explanation see text.

found are plotted in curve $A + V$. Similar tests eliminated the concepts of algebraic summation or geometrical mean, the deviations being beyond the limits of experimental errors. Hunt (*loc. cit.*) obtained approximately the arithmetical mean. This was probably due to his working near the region of intersection of the curves *a. m.* and $A + V$ in figure 9.

We infer that the effects of simultaneous stimulation of the decelerators and accelerators is the *resultant* of the two opposite influences. The two nerves act independently; neither interferes with the other; each exerts its action on the rate imposed by the other as if this were not active.

These results and inferences are consistent with the theory of chemical

mediation of autonomic nerve impulses. Several possibilities could be expected from this theory. For instance, either one of the mediating substances could inactivate the other, or the two could act indepedently on the pacemaker. The experimental data presented confirm the latter suggestion and invalidate the former. The possibility that the rhythm of the denervated heart could be a function of the persistent local metabolic production of sympathin and that vagal stimulation would inhibit this production is thus excluded.

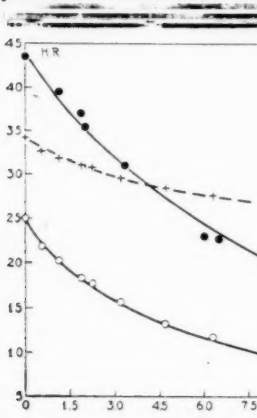

Fig. 9

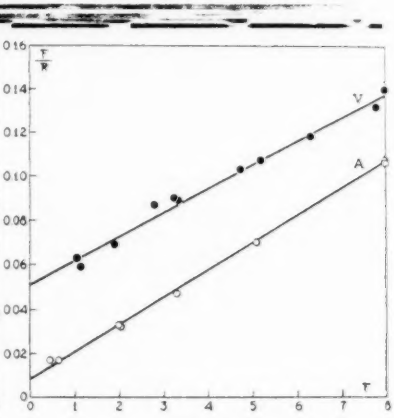

Fig. 10

Fig. 9. Ordinates: h. r. per 10 seconds. Abscissae: frequencies of stimulation applied to the vagus. *V* (circles): vagus alone. *A* + *V* (dots): vagus during persistent stimulation of the accelerator at 8 per second. The curve *a. m.* (crosses) denotes the effects which would have obtained in the latter case if the responses to simultaneous stimulation of the two nerves were the arithmetical mean of the responses to separate activation.

Fig. 10. Test of the curves in figure 12 as hyperbolas. Ordinates: *F/R*. Abscissae: *F*. *V* (dots): vagus. *A* (circles): accelerator. For explanation see text.

The post-acceleration on simultaneous excitation of the two nerves (fig. 5) is readily explained in terms of the rates of destruction of the two mediators; the vagal substance is rapidly destroyed, thus permitting the more stable sympathin to exert its influence. From the data reported in section C we may further conclude that the rates of production and destruction of each of the substances are not modified by the presence of the other mediator.

The post-acceleration has led to the inference that the "point of attack" (*Angriffspunkt*) of the two nerves on the pacemaker is not the same (Tigerstedt, 1921; Asher, 1926). This inference appears to be unwarranted. The opposite effects are probably exerted on the same structure or process,

but one ceases before the other. The problem of the antagonism between the two nerves is intimately related to the preceding concept. Since the influences may be exerted on the same structure and the responses bear opposite signs, the effects would be strictly antagonistic.

The physiological consequences of the tonic presence of the two opposed influences and of their reciprocal decrease or intensification in reflex changes of h. r. in the higher vertebrates may be clarified by the following speculations. Changes of h. r. could be elicited in four ways: *a*, a single nerve supply slowing a fast heart; *b*, a single nerve supply accelerating a quiescent or slow heart; *c*, a dual supply, accelerating and decelerating, respectively, a heart with an average rhythm, and never acting simultaneously, but separately; *d*, finally the actual situation, a dual supply acting constantly, and reciprocally excited and inhibited. The situations *a* and *b* occur in some molluscs (see Tigerstedt, *loc. cit.*, for references). An analysis of the curves in figure 12 leads to the inference that to obtain a given rate with a single nerve supply would require either a higher frequency of discharge or a larger quantum of mediator per nerve impulse (see Rosenblueth, 1932). A dual nerve supply permits, therefore, a finer gradation. In situation *c* recovery from a given slowing or, more specially, acceleration, would be long (cf. curves A and $A + V$, fig. 6). The consequences of the physiological situation *d* are then the possibility of obtaining considerable changes with relatively slow frequencies of nerve discharge, a fine gradation and the possibility of rapid changes.

The significance of the percental scale adopted to estimate the effects of a given stimulus (figs. 2, 4 and 8) instead of the absolute decrement or increment in heart beats per unit time (figs. 1, 3, 5, 6 and 7) will appear from the following considerations. Stimulation of the accelerators or the decelerators does not add to or subtract from the existing rate a certain number of beats; its multiplies the rate by a given factor greater or less than 1; in other words, the degree of acceleration or deceleration obtained is a function of the existing rate on which the stimulus acts. Suppose, adopting a classical view, that the h. r. depends on the length of the refractory period (absolute and relative) and that a given stimulation of the vagus increases this length by a certain amount, say 1 second. Suppose now that this stimulus is applied while the refractory period is 2 seconds. The basal rate of 30 beats per minute will be slowed to 20 during the stimulation; the actual deceleration will then be 10 beats. If the same excitation of the vagus, however, should be performed when the refractory period is 1 second, the rate would go from 60 to 30, an actual slowing of 30 beats per minute. Thus, even if the effects of the vagus should be additive on the refractory period the influence on diverse basal rates would not be additive, but multiplicative. A similar reasoning holds for any other theory adopted to explain the mode of action of the nerves on the h. r. Acceleration and

deceleration, then, not being additive, the concepts of an arithmetic mean or an algebraic summation are *a priori* excluded (see p. 47).

It is interesting to contrast the independence of effects of the excitatory and inhibitory influences in the heart with the dependence of an inhibitory effect on previous excitation in the central nervous system. In the motoneurones, as shown by Sherrington (1925), c.i.s. acts on c.e.s., not on the neurone itself; thus inhibition can only be apparent if excitation is induced. In the heart, on the other hand, the opposed influences are exerted on the pacemaker itself, and vagal effects do not therefore require accelerator tone for demonstration. It is tempting, but premature, to speculate as to whether in neurones that apparently do not require afferent nerve impulses for activation, such as those in the respiratory center, c.e.s. and c.i.s. do not bear relations similar to those found in the heart for the accelerating and decelerating substances.

MATHEMATICAL DISCUSSION. The curves correlating the frequency of stimulation of accelerators or decelerators with the maximum of the corresponding responses at equilibrium, plotted as either absolute (figs. 1 and 3) or percental (figs. 2, 4 and 12) increments or decrements of the h. r. have been previously (Rosenblueth, 1932) shown to be rectangular hyperbolas of the form

$$\pm \Delta R = \frac{F}{k + k'F} \qquad (1)$$

where ΔR denotes the increment (+) or decrement (−); F, the frequency; and k and k', constants.

From (1)

$$\frac{F}{\Delta R} = k + k'F.$$

A simple test of the adequacy of the formula to fit the experimental data is, therefore, to plot $F/\Delta R$ against F; a straight line should obtain. An application of this test to the curves in figure 12 is shown in figure 10.

In order to deal with the effects of simultaneous stimulation of the two nerves, the following formulation is convenient. Let B denote the basal rate of the heart when not influenced by any nerve impulse; R and F, as in (1); and the suffixes a and v, accelerator and vagus, respectively. Then,

$$R_a = mB = B\left[1 + \frac{F_a}{B(k + k'F_a)}\right] \qquad (2)$$

where $m > 1$. And

$$R_v = nB = B\left[1 - \frac{F_v}{B(c + c'F_v)}\right] \qquad (3)$$

where $n < 1$.

If the effects of the two nerves coexist independently,

$$R_{a+v} = mnB \quad \ldots\ldots (4)$$

If now F_a is constant, $m = \alpha$ (constant), then

$$R_{a+v} = \alpha B \left[1 - \frac{F_v}{B(c + c'F_v)} \right] \quad \ldots\ldots (5)$$

And similarly, if $n = \beta$ (constant), then

$$R_{a+v} = \beta B \left[1 + \frac{F_a}{B(k + k'F_a)} \right] \quad \ldots\ldots (6)$$

In (5),

$$\alpha B - R_{a+v} = \frac{\alpha F_v}{c + c'F_v} = \Delta R_{a+v}$$

Hence,

$$\frac{\alpha F_v}{\Delta R_{a+v}} = c + c'F_v$$

That is, the curves *A.i.* (fig. 1) and $V + A$ (fig. 3) should again be rectangular hyperbolas, and $F/\Delta R$ plotted against F should yield a straight line. This test was applied to all the experimental curves with satisfactory results.

The interrelations of the three variables R, F_a and F_v are best appreciated by means of nomograms. By use of the method described in section B an experimental family of curves may be obtained, such as is represented in figure 11. The procedure used involves a large number of determinations, whereupon a certain scattering ensues. The close similarity of this experimental family of curves with the theoretical contour nomogram in figure 13 is, however, striking. In order to construct the theoretical nomograms the values of k and k' and c and c' were calculated from the data in figure 12, obtained in the same animal on which the observations of figure 11 were made. Figure 13 illustrates the h. r. corresponding to variable degrees of stimulation of the vagus, the accelerator being activated with constant given frequencies. If this figure is slabbed by vertical lines and the F_a corresponding to the curves is plotted against the R at the intersection of the line and the curve, the family in figure 14 is obtained, which depicts the responses to variable stimulation of the accelerators with simultaneous activation of the vagi at a given frequency. Vertical slabbing of this figure yields back figure 13. Horizontal slabbing of either of these two families gives figure 15, which shows what frequencies on the two nerves will elicit a given h. r. Vertical slabbing of figure 15 leads back to figure 14, while horizontal slabbing restores figure 13. Angular slabbing through the origin of figure 15 yields figure 16, which represents the responses to various frequencies on the two nerves, the ratio of F_a / F_v being constant.

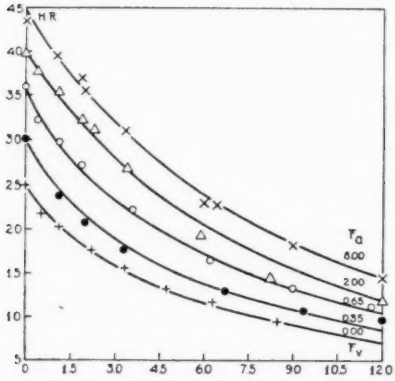

Fig. 11

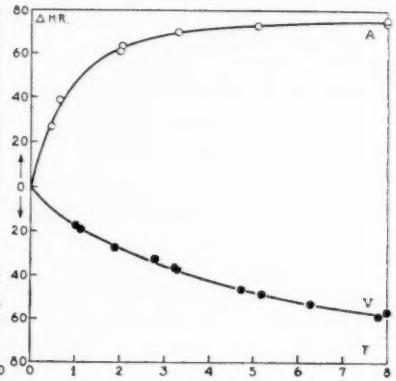

Fig. 12

Fig. 11. Experimental family of curves obtained by means of the method described in section B. Ordinates: h. r. per 10 seconds. Abscissae: frequencies applied to the vagus. The indices of the curves denote the frequency of persistent activation of the accelerator.

Fig. 12. Same animal as in figure 11. Ordinates: percental acceleration and slowing. Abscissae: frequencies. *V* (dots): stimulation of the vagus alone. *A* (circles): stimulation of the accelerator alone.

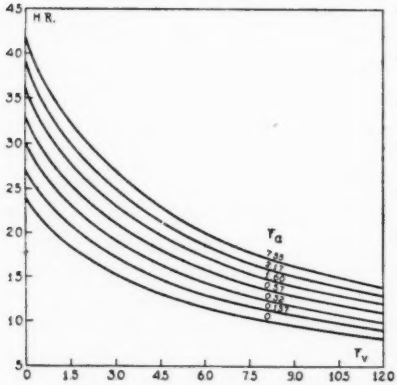

Fig. 13

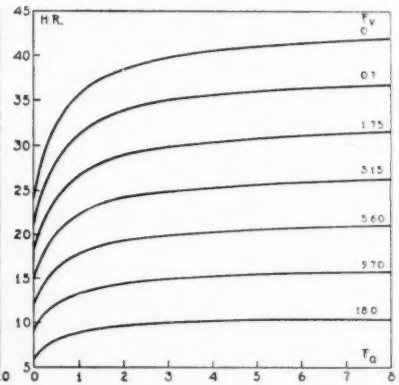

Fig. 14

Fig. 13. Theoretical contour chart. Ordinates: heart rate per 10 seconds. Abscissae: frequency of stimulation of the vagus. Indices of the curves: frequency of stimulation of the accelerator. The basal rate of the denervated heart in this and the succeeding figures is 24 per 10 seconds.

Fig. 14. Theoretical contour chart. Ordinates: heart rate per 10 seconds. Abscissae: frequency of stimulation of the accelerator. Indices of the curves: frequency of stimulation of the vagus.

If the data in figures 13 and 14 are plotted as percental acceleration and deceleration, respectively, figure 12 ensues. This figure shows that the curves of acceleration and deceleration are not continuous. There is a discontinuity at the origin. This is in keeping with the idea expressed above (p. 48), that we are dealing with two independent influences neither of which is similar to the factor responsible for the autonomous rhythm.

The contour nomograms in figures 13, 14, 15 and 16 are advantageous in permitting the selection of the dependent and independent variables. If, however, it is desired to examine the interrelations with new variables, e g., O_2 consumption, heat production, etc., the pictures would become too com-

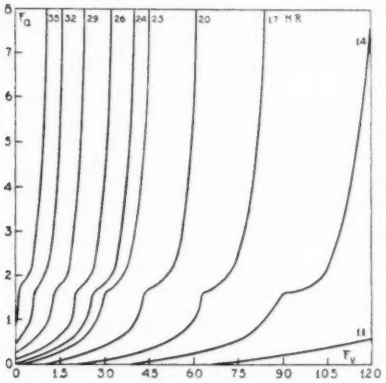

Fig. 15

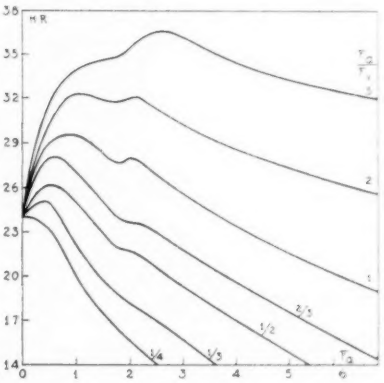

Fig. 16

Fig. 15. Theoretical contour chart. Axes: frequency of stimulation; ordinates, accelerator; abscissae, vagus. Indices of the curves: heart rate per 10 seconds.

Fig. 16. Theoretical contour chart. Ordinates: heart rate per 10 seconds. Abscissae: frequency of stimulation of the accelerator. Indices of curves: ratio F_a/F_v. For explanation see text.

plex for ready interpretation. Figure 17 is a scalar nomogram which summarizes the contour charts. If any two of the variables are selected, the third will be determined by a straight line through the two known values, as usual. The scales on the lines F_a and F_v are hyperbolic, that on H. R. is logarithmic. To this nomogram an indefinite number of scales may be added, representing new correlated variables, such as those mentioned above.

The preceding formulation covers only temporal variations of the stimuli applied to the nerves. Spatial variations can, however, be dealt with by means of the same nomograms. The scales marked F can denote N (number of nerve impulses per unit time), for $N = nF$, where n is the number

of nerve fibers involved (see Rosenblueth and Rioch, 1933). In the experiments here reported n was kept constant by using maximal stimuli.

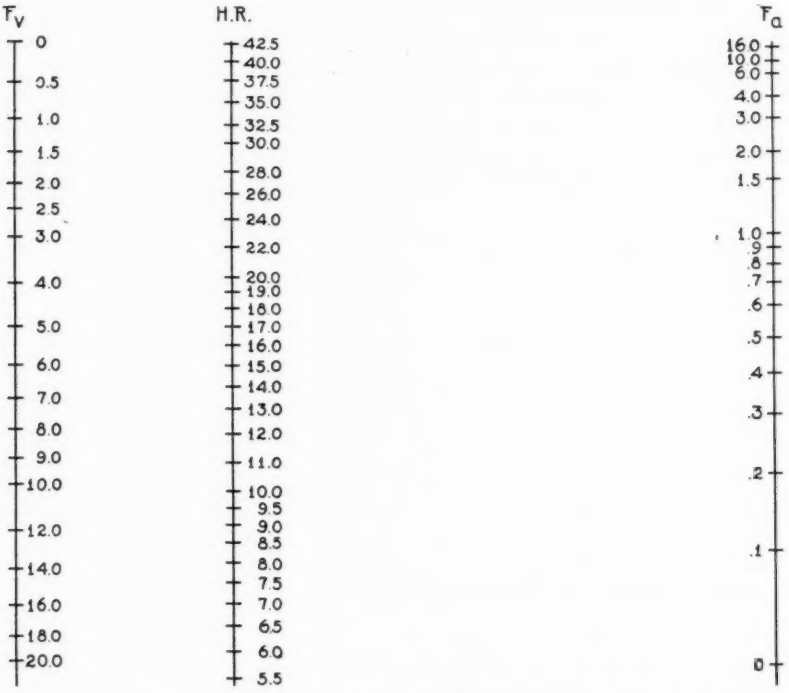

Fig. 17. Scalar nomogram. F_a: frequency of stimulation of the accelerator. *H. R.*: heart rate per 10 seconds. F_v: frequency of stimulation of the vagus.

SUMMARY

The changes of heart rate at equilibrium on stimulation of the vagus at various frequencies, with and without a simultaneous constant accelerator tonic discharge (fig. 1), are similar for any frequency on the vagus when plotted as per cent of the corresponding basal rate (fig. 2).

The maximal percental effects of a given vagal stimulation are likewise the same (fig. 4) no matter what simultaneous activation of the accelerators is applied (figs. 3 and 11).

The independence of the percental effects on stimulation of either nerve from the simultaneous activation of the other exists not only at equilibrium, but throughout the time course of the responses (figs. 5, 6, 7 and 8; section C).

It is concluded that the effects of simultaneous excitation of the accelerators and decelerators is not the arithmetical mean (fig. 9), nor an algebraic summation, nor the geometrical mean of the responses to separate stimulation (p. 47), but the *resultant* of the two influences. The two effects occur independently, as if each set of nerves was acting alone.

This conclusion is discussed in relation to the following subjects: the chemical mediation of antonomic nerve impulses (p. 48), the "point of attack" of the two nerves on the pacemaker (p. 48), the implications of the tonic activity of the two nerves and the changes which occur in cardiac reflexes (p. 49), the multiplicative—not additive—nature of acceleration and deceleration (p. 49), and the interrelations of excitation and inhibition in the central nervous system (p. 50).

A mathematical discussion is presented (p. 50). This includes a graphic representation (figs. 13, 14, 15, 16 and 17) which correlates the variables involved.

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THE EFFECT OF INJECTION OF MONOIODOACETIC ACID AND SODIUM CYANIDE ON THE MAMMALIAN HEART

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Changes in the S-T segment of the electrocardiogram of man and experimental animals have come to be associated with a deficient supply of oxygen to the ventricular myocardium (Randles, Gorham and Dresbach, 1). Kountz and Hammouda (2) have suggested that such alterations result from the accumulation of high concentrations of locally produced metabolites. In experimental coronary occlusion, where such electrocardiographic changes are most uniformly observed, Himwich and his co-workers (3) have demonstrated that high concentrations of metabolites are in fact found in the infarcted areas.

Since poisoning with monoiodoacetic acid would prevent the accumulation of lactic acid while poisoning with cyanide would promote such accumulation, the following experiments were undertaken to study the effect on the electrocardiogram of poisoning with monoiodoacetic acid and sodium cyanide.

METHOD. Cats were anesthetized by intraperitoneal injections of 40 to 60 mgm. of sodium amytal per kilo, and prepared for continuous electrocardiographic recording from lead II (Nahum and Hoff, 4). Artificial respiration was maintained through a tracheal cannula, care being taken to avoid overventilation.

In some experiments oxidations were blocked by subcutaneous injections of sodium cyanide in doses of 25 to 50 mgm. per kilo and in others glycolysis was inhibited by intravenous injections of 50 to 150 mgm. per kilo of monoiodoacetic acid neutralized with sodium carbonate.

RESULTS. A. *Monoiodoacetic acid.* The first change to follow injection of monoiodoacetic acid was a marked increase in heart rate and a decrease in intracardiac conduction times. In figure 1, A and B, which is from a typical experiment, the rate increased from 128 to 205, the P-R interval shortened from 0.08 to 0.06 second, and the Q-T interval from 0.24 to 0.18 second. The T wave changed from positive to negative.

From this point, the rate as well as the rhythm remained remarkably constant. In some experiments A-V block and disappearance of the P wave occurred shortly before arrest of the ventricle, though in the others, as in figure 1, the auricle continued to beat, and A-V conduction remained

unchanged to the very moment of complete cardiac standstill. This is illustrated in figure 1, G, which shows the terminal complexes. In this as in all experiments, the heart stopped suddenly. Following the rapid

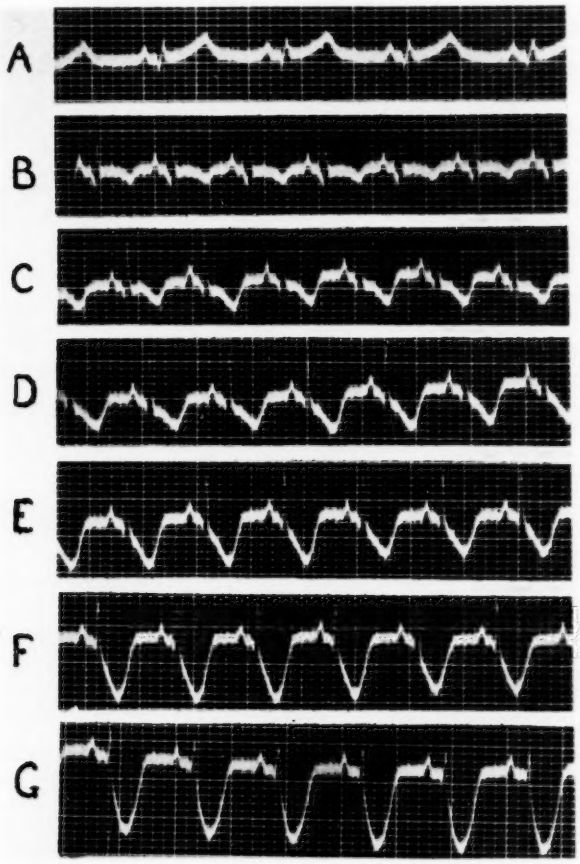

Fig. 1. *Experiment 108.* May 17, 1934. Cat 2.5 kgm. Sodium amytal anesthesia. Electrocardiogram from lead II. Artificial respiration after A.

A. Control. B. One minute after an intravenous injection of 200 mgm. monoiodoacetic acid neutralized with sodium carbonate. C, D, E, F and G. Records at intervals of two minutes. Complete cessation of heart beat immediately after G.

initial decrease in contraction time, there occurred a further more gradual decrease which is shown in figure 1, as a shortening of the Q-T interval from 0.18 second in B, to 0.15 second in G.

In addition to the increase in heart rate and decrease in conduction time, the predominant electrocardiographic feature was a progressive alteration

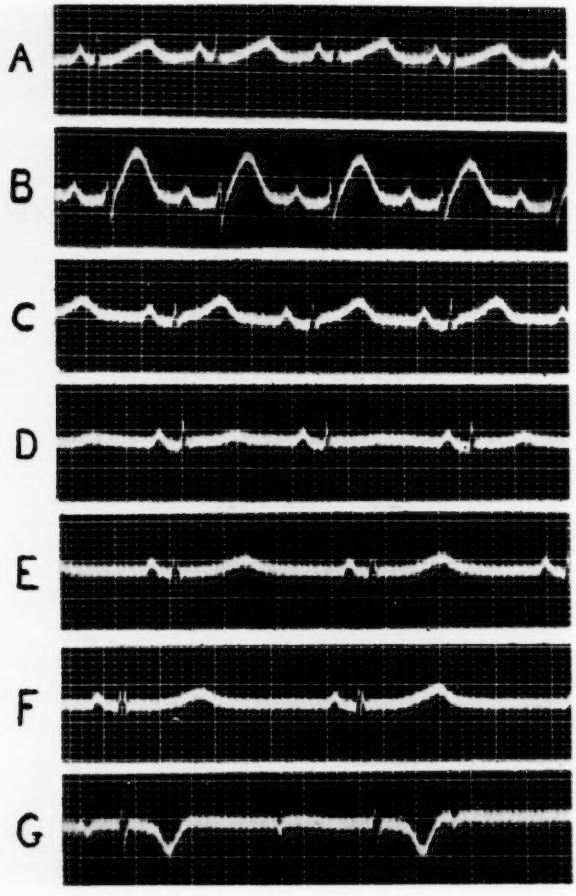

Fig. 2. *Experiment 106.* May 15, 1934. Cat 2 kgm. Sodium amytal anesthesia. Electrocardiograms from lead II. Artificial respiration after A.

A. Control. B. One minute after subcutaneous injection of 150 mgm. sodium cyanide. C, D, E and F. Records taken at two minute intervals. G. Terminal record from another experiment showing complete A-V block.

of the S-T segment. This consisted in a displacement of the S-T segment below or above the isoelectric line, and in a simultaneous increase in the height of the T wave, with disappearance of the isoelectric interval. These

events are illustrated in figure 1 where the displacement of the S-T segment is first noticeable in C, and becomes more and more marked in D, E, F, and G. In the last record the S-T segment starts at least 4.5 mm. below the isoelectric line. In C, there still exists an isoelectric period in the S-T segment, although it is much reduced, and in D it has completely disappeared. The gradual enlargement of the T wave is also clearly shown.

B. *Cyanide.* The electrocardiographic effects of cyanide poisoning were almost diametrically opposite to those of monoiodoacetic acid. Following a transient effect on the S-T segment, occurring only with larger doses (see fig. 2B), the main features were slowing of rate, delay in A-V conduction leading to A-V block (fig. 2 G), and a prolongation of the ventricular complex without essential change in the S-T segment. Auricular standstill took place before ventricular arrest, and the final ventricular complexes showed perfectly normal S-T segments.

Discussion. The essential changes induced by monoiodoacetic acid are 1, an increase in heart rate, a decrease in conduction time, and a shortening of the ventricular complex, and 2, a progressive alteration in the S-T segment, resembling closely the changes which occur in conditions associated with anoxemia of the myocardium (Randles, Gorham and Dresbach, 1). Goldenberg and Rothberger (5) and Dobrowalski (6) have recently reported similar changes in the dog following poisoning with monoiodoacetic acid. (See also De Boer and Spanhoff, 7.)

In the doses employed monoiodoacetic acid is known to block glycolysis completely, without suppressing the oxidative mechanisms or the anaerobic cleavage of phosphagen. In such conditions phosphoric acid and creatine would tend to accumulate while lactic acid could not. The electrocardiographic changes reported in this study can not therefore be due to the accumulation of lactic acid, but are evidently produced by the loss of the energy usually derived from the breakdown of glycogen.

The explanation for the acceleration of rhythmic, conducting and contractile processes, invariably found in poisoning with monoiodoacetic acid must be in some way related to the loss of glycolytic energy in all the structures of the heart. The maintenance of a more or less constant, rapid heart rate during the period of progressive S-T segment changes, accompanying the loss of anaerobic energy suggests strongly that the rhythmic process has an aerobic basis. Since the metabolic demands of the contracting ventricle are greater than those of the auricle, and very much greater than those of the pacemaker and conducting systems, the ventricle is the first to fail. In addition, as suggested above, the neuromuscular system of the heart might depend to a greater extent upon aerobic energy for its metabolic needs, and for this reason also may survive for a longer period. That such is indeed the case is shown by the experiments in which the aerobic oxidations were suppressed by cyanide.

In cyanide poisoning oxidations alone are suppressed while the activity

of anaerobic systems is unimpaired. In these circumstances the concentrations of lactic acid as well as phosphoric acid and creatine increase, yet no permanent alterations occur in the ventricular complex of the type which has been recognized as due to myocardial anoxemia. Accumulation of disintegration products of phosphocreatine could not therefore have been the cause of the changes in the S-T segment found in monoiodoacetic acid poisoning, since they must occur also in cyanide poisoning without S-T changes. In general it can thus be inferred that accumulation of metabolites alone plays but a small part in the production of changes in the ventricular complex.

The predominant electrocardiographic characteristic of cyanide poisoning is damage to the rhythmic and conduction systems. It would thus appear that the pacemaker and conduction tissue are more dependent on aerobic than anaerobic metabolism. The contractile mechanisms on the other hand show practically no disturbances when aerobic metabolism is blocked but change profoundly when glycogen breakdown is prevented.

It seems therefore that the rhythmic and conductile tissues of the heart resemble the nervous system in their greater dependence on aerobic metabolism, while the myocardium operates effectively on an anaerobic basis.

SUMMARY. 1. The failure of the anaerobic energy system of the heart induced by monoiodoacetic acid leads to an acceleration in heart rate, a decrease in conduction time, and a reduction of the duration of the ventricular complex, as well as to profound alterations in the ventricular complex characterized by a change in the direction of the T wave, disappearance of the isoelectric interval, and displacement of the S-T segment.

2. The suppression of oxidations by cyanide results in marked slowing of the heart rate, gradual development of A-V block, and prolongation of the duration of the ventricular complex without further permanent changes in the ventricular complex.

CONCLUSION

The pacemaker and conducting tissue of the heart operate essentially on aerobic energy, while the myocardium is primarily anaerobic. Changes in the S-T segment of the electrocardiogram in these experiment are related, not to accumulation of metabolites, nor to failure of oxidations, but to a loss of the anaerobic energy of glycogen breakdown.

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THE OXYGEN CONSUMPTION OF RABBIT BONE MARROW IN RELATION TO ITS MORPHOLOGY

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Few direct measurements have so far been made of the metabolic activity of bone marrow. Soru and Brauner (1931) measured the oxidation-reduction potential of rabbit bone marrow by inserting small electrodes into that tissue in living rabbits and noting the potential developed. This they did before and after the development of a secondary anemia, and they found that the rH was slightly higher in the case of the anemic animals. Felix, Grassmück, Huck and Matzen (1933) measured the oxygen consumption of calf marrow manometrically. They obtained widely different values for different samples of marrow, but found there was a rough correspondence between the oxygen consumption and the purine nitrogen content of the marrow. The latter figure may be considered to be an approximate index of the total nuclear content of the marrow, or, if we disregard the fact that in the marrow many of the cells are non-nucleated, of its total cellular content. Since this is probably the basis upon which the relation rests, it is not surprising to find that when the oxygen consumption is expressed in terms of cc./mgm. purine N/hr., the values still vary over more than a six-fold range. As these authors themselves point out, the oxygen consumption must be related to the different types of cells present as well as to their total numbers. It is with just such a relation that the present paper deals, although the work was completed at the time of publication of the paper just referred to. I have used counts of marrow smears to determine the relative numbers of the different classes of cells present, and special methods for measuring the rate of oxygen consumption. The paper is divided into two parts. Part I deals with the method developed for measuring the oxygen consumption of the marrow, and with certain preliminary experiments designed, for the most part, to test this method. In part II the oxygen consumption of the marrow is related to its histological composition.

PART I. A. *Methods and preliminary experiments.* Probably the principal reason why metabolic studies of bone marrow have been slow to appear is that the tissue is an awkward one to handle. It is usually very soft and diffuse, which makes proper cutting difficult, and its composition is so variable, even in a single bone, that special methods must be used to obtain

even a small number of samples which are comparable. In the method to be described, both these difficulties are to a large extent overcome.

The animals are killed by venesection and the femora are at once removed and scraped clean of adhering tissue. The knee joint is cut off and the bones are carefully cracked with a nut-cracker in such a way that the marrow is not squeezed out. The fragments of bone on one side of the femur are removed, and the marrow is removed as a "pencil" by freeing the endosteum from the bone with a blunt instrument. With sufficient care this can be done in most cases without seriously puncturing the endosteum.

The best way of imbedding the marrow for cutting is to use tubes of paraffin with internal diameters of different sizes. These tubes are cut with cork-borers from paraffin blocks. The outside diameter is about 1 cm. and the tubes are about half an inch longer than the marrow itself. Most marrows when obtained as described above are found to have a slight taper. A paraffin tube is selected which has an internal diameter about equal to that of the smaller end of the marrow; this end is inserted into the opening and a gentle suction applied to the other end of the tube. By holding the preparation upright and gently manipulating the marrow sideways one can make it slide snugly into the paraffin tube leaving about a half inch of the tube unfilled. This part is gently warmed and compressed, closing that end off completely, and the marrow is thus encased and ready for cutting in a hand microtome. In these experiments the thickness of each slice is 500 μ, a matter which will presently be discussed more fully. About nine serial sections are obtained on the knife blade at one time, the encircling paraffin is knocked off, and the slices are transferred alternately to each of three Thunberg-Winterstein respirometers by means of a camel's-hair brush. If the fluids into which the tissue slices are placed are different, a separate brush is used for each respirometer to prevent contamination. As a rule, a dozen or so slices are placed in each respirometer; they represent a series of samples each 500 μ thick taken at 1.5 mm. intervals down the marrow. That the contents of the respirometers are really comparable will presently be demonstrated. The whole procedure is carried out under essentially aseptic conditions and requires about a half-hour from the time the animal is killed until the respirometers are placed in the water-bath for a period of twenty minutes equilibration.

The method of respirometry has been fully described by Fenn (1927), and such details as apply to the use of the apparatus in the present work have been given by Ramsey and Warren (1930). The measurement of oxygen consumption is continued for a period varying from 20 minutes to an hour and a half depending on the activity of the tissue. During this time the rate of oxygen consumption decreased by an amount varying between 5 and 18 per cent. At the end of this time the contents of the respirometers are transferred quantitatively to porcelain crucibles and the

weight taken after drying to constant weight at 60°, since higher temperatures are apt to volatilize the fats present. A deduction is made for the dry weight of the solutions used. The oxygen consumption is then expressed as mm.^{3} O_2 (at 37°)/dry grm./hr.

It has long been realized that in studies of microrespiration the slices of tissue must be thin enough so that oxygen and metabolites shall have free access to all parts of the tissue by diffusion. The limiting thickness, granted adequate shaking, can be calculated by the formula of Warburg (1926), but two of the constants which must be known are the rate of oxygen consumption of the tissue and its diffusion constant for oxygen, and in the present case these values were not available at the outset. Consequently, measurements of oxygen consumption were made in which the thickness of the slices in the different respirometers varied. The results

TABLE 1

Effect on the oxygen consumption of varying the thickness of the slices of tissue

Oxygen consumption in mm.^{3}/grm./hr.

EXPT.	SIZE OF SLICE					SHAKEN ONLY	MAX. DIF.
	250 μ	400 μ	500 μ	800 μ	1200 μ		
							per cent
1			1200			800	33
2			445			256	33
3			712			555	22
8		465		465	440		5
9		517		515	412		20
29	1240		1290				4

are given in table 1, which shows that although simply disintegrating the marrow by shaking it is not satisfactory (since it gives values about 30 per cent too low), the thickness of the slice can nevertheless vary from 250 to 800 μ without the respiration being affected. At a thickness of 1200 μ, however, one of two experiments shows a decreased respiration. Special attention should be directed to experiment 29 in which a particularly active marrow was selected and the thinnest slices of all were obtained. Even under these circumstances the rate of respiration is the same as that of the 500 μ slices. Slices of the latter thickness are relatively easy to obtain and have been used throughout the following experiments.

To further confirm the conclusion that 500 μ slices are thin enough, four experiments were performed in which the air in the vessels was replaced by oxygen. If, in air, the oxygen were not reaching the center of the slices, higher rates of respiration should be found in an atmosphere of oxygen. Actually, however, the two sets of values were the same.

One may next inquire into the consistency with which a given figure for O_2 consumption can be obtained. In table 2 are listed six experiments on the oxygen consumption of different marrows, five of which are in triplicate and one in duplicate. The last column shows that the greatest difference between any two readings in a single experiment was 8.5 per cent with an average maximum difference of 5.1 per cent. This degree of accuracy is just about the same as may be expected when the three samples are known

TABLE 2

Oxygen consumption measurements in triplicate

EXPT.	OXYGEN CONSUMPTION IN MM.^{3}/GRM./HR.			MAX. DIF.
	A	B	C	
				per cent
4	765	765	763	—
5	625	640	590	6
20	466	495	477	6
21	715	—	755	5.5
25	129	136	130	5
27	1270	1190	1300	8.5

TABLE 3

Oxygen consumption of normal rabbit bone marrow

EXPT.	MARROW	OXYGEN CONSUMPTION MM.^{3}/GM./HR.	EXPT.	MARROW	OXYGEN CONSUMPTION MM.^{3}/GM./HR.
2	2-A	445	15	10-A	555
3	3-A	712	17	11-A	760
4	4-A	765	19	12-A	660
6	5-A	785	20	13-A	479
8	6-A	465	21	14-A	735
9	7-A	850	25	16-A	132
11	8-A	205	27	17-A	1250
13	9-A	550	Average.....		622

to be identical (as in the case of measurements of the oxygen consumption of blood) and is a verification of the accuracy of the method of sampling.

B. *Values of oxygen consumpton for normal rabbit bone marrow.* In table 3 are collected the results of 15 experiments on the oxygen consumption of normal rabbit bone marrow. The variations between different marrows are very apparent, and the average figure is accordingly without particular significance.

PART II. A. *Methods employed in the histological studies.* In the experiments now to be described the oxygen consumption was measured by the

methods described above, but two additions to the procedure were made in order to obtain information regarding the morphology of each particular marrow. These are: 1, counts of the various types of cells present, and 2, an estimation of the fat content in each case.

1. *Cell counts.* Every fifth slice of marrow is set aside as a cytological sample and is placed in a few drops of isotonic buffer (pH 6.4) contained in a small mortar. These samples are then ground lightly with a pestle, and after thorough mixing, dry smears are made of the resulting suspension. Five slides are prepared from each marrow. These are fixed and stained with Jenner and Giemsa as described by Climenko (1930). The slides are ready for examination before the oxygen consumption measurements are completed.

At least 2000 cells are counted and divided into two classes, myeloid and erythroid. Connective tissue cells, histiocytes and lymphocytes are not counted, as ordinarily they are present in too small a proportion of the total cells to seriously influence the results. Neither are the mature erythrocytes counted, for they are really not constituents of the marrow at all, and their oxygen consumption is so small as not to affect the total except in special cases which will be considered individually. The myeloid-erythroid ratio so determined is expressed numerically as the percentage of the total number of cells which are myeloid. As will be seen below, this is an important index of the metabolic activity of the marrow. It is now important to inquire whether the extent to which the tissue is ground influences the myeloid-erythroid ratio. I have repeatedly investigated this point and have invariably found that the relative amount of grinding makes extraordinarily little difference, so small, in fact, that it would be difficult to establish whether any such exists. Severe grinding leads, however, to sufficient injury to the cells to render them less suitable for staining, and it is for this reason that I have avoided grinding more than the minimum amount necessary to secure sufficient breakdown of the tissue.

An idea of the accuracy of the counts may be obtained as follows. In each experiment, the count of 2000 cells was made in four groups of approximately 500 cells each. The myeloid-erythroid ratio was computed for each group and the four results averaged to obtain a figure for the particular marrow under consideration. These averages were compared with the myeloid-erythroid ratios found for the constituent groups of 500 cells and it was discovered that the latter fell within a range which averaged $\pm$ 5 per cent of the final figures. These final figures themselves may accordingly be considered to be accurate to within a considerably more restricted range, say $\pm$ 3 per cent. This is an expression of the counting error inherent in the method, but it fails to take into account another error in the determination of the myeloid-erythroid ratio. This error lies in the relatively large masses of tissue which resist almost any amount of

grinding. In the larger of these, the cells cannot be properly counted, and the masses seem to be predominantly myeloid. For this reason the counts given in this paper may or may not represent fairly close estimates of the actual cellular composition of the marrow, but they are certainly comparable with one another. They also correspond very closely to the figures of Sabin and Doan (1927). A distinct advantage of this method is that the slides represent fairly well-distributed samples taken from the marrow at large, and not from a small isolated portion.

Having determined the myeloid-erythroid ratio, the next step is to count 500 cells in each class to subdivide them again with respect to the degree of their maturity. In each series, an arbitrary stage of development was selected as the dividing line between young and mature forms. In the myeloid line, I have made this the premyelocyte, which is readily distinguishable by its grey color and small granules. In the erythroid series, all cells other than relatively mature normoblasts are counted as young cells. In this instance, the degree of differentiation of the nucleus is of considerable importance. If, in the nucleus, there is a clear demarcation between the oxy- and basichromatin, particularly if the nucleus shows a radial arrangement, the cell is counted as immature; if there is little or no differentiation in the nucleus, the cell is considered to be mature. The results of these counts, in both series, is expressed numerically as the percentage of cells in each line which are immature.

In addition to making marrow counts, red cell, white cell and reticulocyte counts were made on oxalated samples of the blood of each animal. The red and white cell counts were made in the usual way, and reticulocytes were determined in wet mounts. The results of the blood cell counts will not be given in the tables below, because no significant relation was found to obtain between any blood cell count and the oxygen consumption of the marrow.

2. *Fat and lipoid content.* In this instance also, the method was selected for its simplicity rather than for a high degree of accuracy. The same samples of marrow were used in this case as were used for the measurement of the oxygen consumption. The dry weight was first determined as mentioned in part I and these dried samples were transferred to 250 cc. flasks and refluxed for 4 hours with a 1:1 mixture of chloroform and ether. The mixture was then filtered into weighed evaporating dishes, the filter paper washed with ether, and the filtrate dried overnight at 110°, the extracted fats and lipoids being determined gravimetrically. The procedure was first tested to show that no higher yields were obtained when the period of extraction was increased beyond 4 hours. The whole manipulation was standardized with respect to time and the resulting estimations were considered to be comparable with each other, although probably not strictly accurate in the quantitative sense. The determinations were made in duplicate, and the results customarily agreed to within 2 per cent.

B. *Results.* Nineteen experiments in all were performed, and for purposes of analysis they are divided into four groups as follows:

TABLE 4

Experimental data for part II

(1)	(2)	(3)	(4)	(5)	(6)	(7)
EXPT. NO.	ANIMAL	OXYGEN CONSUMPTION MM.^{3}/GRM./HR.	MYELOID-ERYTHROID RATIO $\frac{\text{MYELOID}}{\text{TOTAL}} \times 100$	PER CENT ERYTHROID CELLS IMMATURE	PER CENT MYELOID CELLS IMMATURE	PER CENT FAT
		Group I—Normal (⊙)*				
33	6 lb. grey	323	54	20	30	—
35	4¼ lb. grey	1,000	75	11	20	—
36	4 lb. albino	342	53	26	35	—
38	5½ lb. grey	350	49	22	13	88
39	5¼ lb. grey	470	66	24	36	78
40	6 lb. black	1,045	83	34	17	—
41	5 lb. albino	465	62	29	32	75
42	4½ lb. grey	880	70	68	8	80
43	5 lb. grey	540	69	15	16	75
45	6 lb. albino	443	74	49	15	79
46	8 lb. albino	970	80	20	14	85
Average		620	72	29	22	84
		Group II—Intensely granular (△)				
47	8½ lb. albino	918	63	13	48	86
48	7½ lb. albino	942	65	18	23	83
Average		930	64	15	35	85
		Group III—Young, with erythroid reaction (⊡)				
49	3¾ lb. brown	1,200	29	81	40	72
50	4 lb. brown	2,025	65	91	32	70
51	4¼ lb. brown	770	47	70	47	87
52	4¼ lb. grey	1,155	56	90	33	84
Average		1,288	49	83	38	78
		Group IV—Abnormal (+)				
37	6½ lb. brown	1,000	50	56	40	78
44	5¾ lb.	262	88	12	26	90

* The symbols given after each grouping refer to those used in plotting the data in figure 1.

Group I (11 expts.). Normal mature animals in which the marrow counts reveal a state of maturity in the cells of both the myeloid and erythroid series.

Group II (2 expts.). Normal mature animals in which the myeloid forms, in addition to being rather less mature than the erythroid cells, show an exceptionally marked degree of granulation.

Group III (4 expts.). Immature animals which show a preponderance of immature erythroid cells.

Group IV (2 expts.). Abnormal animals which will be discussed individually.

In table 4 the data pertaining to these experiments are listed, and in figure 1 the oxygen consumption is plotted with respect to one variable,—the myeloid-erythroid ratio.

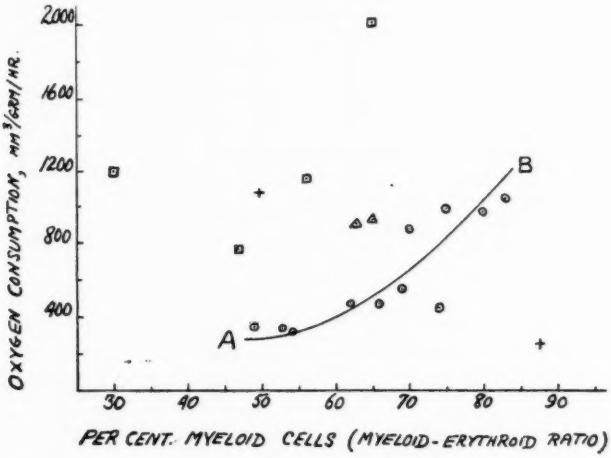

Fig. 1. The differentiation of the points is with respect to the different groups of experiments. Group I, circles. Line *AB* is drawn with reference to these points only. Group II, triangles; group III, squares; group IV, crosses. For explanations of the groupings see text.

Group I. Normal mature animals in which the marrow counts reveal a state of maturity in the cells of both the myeloid and erythroid series.

When the differential counts in both the myeloid and erythroid lines indicate essentially that a state of maturity exists in the marrow, the variable which principally determines the rate of oxygen consumption is the myeloid-erythroid ratio. The higher this ratio, the greater is the oxygen consumption. Line *AB* in figure 1 is an approximation drawn through the points included in this group (circles) and the upward trend of the line bears out this statement. As would be expected when the points are plotted with respect to only one of the numerous variables, a number of the points in this group diverge seriously from the line. In some cases

an explanation of this deviation can be found in the data, but I am compelled to remark that there are many deviations from the general rules which had best be admitted to be unexplained. The line AB can obviously not be considered an accurate representation of the facts in any quantitative sense. My only conclusion with respect to it is that its slope is certainly upwards, and this is so obvious as not to require further discussion.

We may accordingly consider that the marrow of mature normal rabbits, in which there is a relatively low myeloid-erythroid ratio, with only small numbers of immature forms in either series, represents a basal level of metabolic activity of the marrow. These marrows exhibit the lowest rate of oxygen consumption to be found in normal marrow. This low metabolic rate is increased if either or both of two histological changes occur. In the first place, the myeloid-erythroid ratio may increase without significant changes in the proportion of immature forms present. This will increase the rate of oxygen consumption in a direction which is shown by the line AB of figure 1. Secondly, there may be important increases in the proportion of immature cells. This, and other subsidiary changes, will also increase the basal metabolic rate, and these effects will be described below.

Group II. Normal animals in which the myeloid line is prominent and characterized by intense granulation in the myelocytes.

These marrows exhibit a high rate of oxygen consumption out of all proportion to what might be expected (in view of the results of group I) from their moderately high myeloid-erythroid ratio. Cytological examination reveals very little erythroid activity and the proportion of young myeloid cells, although high in one case, is not high in the other. But the slides show one feature in common,—the myelocytes are exceptionally well developed and their cytoplasm contains an unusual number of intensely-staining large granules. These cells are apparently the cause of the high oxygen consumption. Furthermore, examination of the slides in all groups reveals the fact that in the myeloid series it is the extent of granulation, and not the age of the cell, that primarily determines the rate of oxygen consumption. This is a fact which does not lend itself to quantitative treatment, but examination of the results in group I discloses that there is no marked relation between the percentage of young myeloid cells (column 6) and the oxygen consumption. This result is in contrast to that which applies to the erythroid line, for in the latter case, since the cells contain no granules, the degree of maturity is the distinguishing characteristic by which the oxygen consumption may be forecast.

Group III. Young animals in which the marrow contains a large proportion of immature erythroid cells. The marrow of young rabbits exhibits a predominantly erythroid condition, as evidenced both by the increased proportion of erythroid cells (a low myeloid-erythroid ratio) and a preponderence, within this group, of immature forms. Many young

myeloid cells are also present, but their proportion is not increased to anywhere near the same extent as in the case of the immature erythroid cells. These marrows are exceedingly active, as the distribution of the points (squares) on figure 1 indicates. From these facts the obvious conclusion is that the high rate of oxygen consumption is due to the preponderance of young erythroid forms. This is supported by the observation that the marrow in this group which was least active (expt. 51) was the one having the smallest proportion (70 per cent) of these cells, whereas in that showing the highest rate of oxygen consumption (expt. 50) the percentage of young erythroid forms was highest (91 per cent). The oxygen consumptions in this group vary over a wide range, but despite this I believe the experiments clearly demonstrate that the immature erythroid cell possesses a high rate of oxygen consumption. The very high proportion of these cells (83 per cent as against 29 per cent in group I) combined with a relatively low myeloid-erythroid ratio and only a small increase in the usual proportion of immature myeloid forms indicates clearly that the young erythroid cell is an active one metabolically, at least in so far as its rate of oxygen consumption is concerned.

Group IV. Abnormal animals. The first of these (expt. 37) was a healthy animal which had been injected over a period of 16 days with 0.5 gram of thym-nucleic acid. A leucocytic reaction of the granular type was evident in the blood, the progressive shift to young forms suggesting a leucogenic reaction in the bone marrow. Erythrogenic activity was also indicated by a fairly high reticulocyte count (2.5 per cent).

This marrow proved to have a high rate of oxygen consumption and it was interesting to discover that in the marrow the erythrogenic reaction was much more pronounced than the leucogenic one. Both with respect to its oxygen consumption and to its cytology, the marrow closely resembled that of a young animal such as has been described in the preceding section.

The animal of experiment 44 was found, at mortem examination, to be suffering from lobar pneumonia in the stage of grey heptization, and from an acute enteritis.

The marrow of this animal, on gross examination, appeared to be composed almost entirely of a fatty gelatinous material. This was also evident from the slides, and great difficulty was experienced in finding the requisite number of cells for counting. Neither the counts themselves, however, nor the high percentage of fat found by analysis seem to me to accurately describe the almost completely aplastic condition of the marrow. Quite probably a very large proportion of the non-fatty material consisted of gelatinous matrix.

In view of these cytological findings it was not surprising to discover that this marrow consumed oxygen at the lowest rate of all those studied.

Owing to the high myeloid-erythroid ratio, the divergence of this marrow from the normal group is clearly evident in figure 1.

Discussion. The average oxygen consumption of the 11 normal marrows of group I is 620 mm.^{3}/grm./hr. and that of the 15 normal marrows of part I is 622 mm.^{3}/grm./hr. The fortuitous agreement between the results of these two groups of experiments is of no significance, but normal rabbit bone marrow must be considered as a tissue having a comparatively low respiratory activity, for even a rate of 1000 mm.^{3}/grm./hr. is only $\frac{1}{7}$ that of excised renal tissue of the dog, $\frac{1}{30}$ that of rat kidney and $\frac{1}{10}$ that of rat testis (Richardson, Shorr and Loebel, 1930), $\frac{1}{3}$ that of resting dog muscle (Himwich and Castle, 1927), and about $\frac{1}{10}$ that of rat liver or of Flexner rat carcinoma (Warburg, 1926). It is, however, about equal to the oxygen consumption of resting rabbit nerve (Gerard, 1930), slightly greater than the oxygen consumption of rabbit reticulocytes and 40 times as great as that of mature rabbit red cells (Ramsey and Warren, 1934). The rates of oxygen consumption of calf marrow obtained by Felix, Grassmück, Huck and Matzen (1933) lie within the range of values obtained here for rabbit marrow.

In considering the results of part II, there are two facts established by the experiments. The first of these is that with normal adult marrows the rate of respiration increases as the proportion of myeloid cells (the myeloid-erythroid ratio) increases. This appears at first sight to indicate that the average erythroid cell is considerably less active, metabolically, than the average myeloid cell, and in a certain sense this is true. But the difference in metabolic activity between these two types of cells becomes considerably less marked when one considers their difference in size. In dry films it is impossible to measure differences in cell volume accurately because, in addition to the cells being of irregular outline, it is a question whether they are essentially discs or spheres. Nevertheless, I have measured the diameters of 150 cells of both classes chosen at random from marrows of normal animals. The average diameter of the myeloid cell is about twice that of the average erythroid cell, and its average volume must be at least four times greater, so even the relatively mature erythroid cell exhibits an oxygen consumption which, in proportion to its volume, is not far removed from that of the average myeloid cell. This result is not surprising in view of the fact that reticulocytes consume oxygen 30 times more rapidly than the mature erythrocyte (Ramsey and Warren, 1934). If the presence of a small amount of cytoplasmic differentiation leads to such a marked change in metabolic activity, it is not surprising to discover that in the presence of a nucleus, the cell becomes an even more active one.

The second outstanding point is the demonstration that the relatively immature erythroid cell unquestionably exhibits a high rate of oxygen consumption. This result extends, and to a certain extent confirms the remarks made in the preceding paragraph.

The conclusion drawn from the experiments of group II, viz., that in the myeloid series it is the extent of granulation, more than the age of the cell, which is important in determining its oxygen consumption, should be considered as a preliminary suggestion rather than as a well-established fact. Both the qualitative nature of the observations and the small number of experiments in this group demand this qualification, although such a result would not be unreasonable in view of the rather general association of high metabolic activity with marked cytoplasmic granulation. It is also indirectly supported by the work of Soffer and Wintrobe (1932) who measured the oxygen consumption of leucocytes in normal and leucemic blood and found no significant relation between the degree of maturity of the cells and their oxygen consumption. Further studies are required, however, to fully describe the state of affairs in this particular case.

The significance of the experiments of group IV on abnormal animals lies in the demonstration that changes in the marrow such as may occur in pathological conditions are readily detectable by the methods described. It is not likely that such measurements of oxygen consumption by themselves will yield significant information as to the metabolic basis of pathological conditions, but when supplemented by other metabolic measurements the results may prove of interest.

SUMMARY

1. A method of preparing rabbit bone marrow for the measurement of its oxygen consumption is described.

2. The oxygen consumption of the marrow of normal adult rabbits is variable, but averages about 600 mm.^{3}/dry grm./hr. The variations are due to differences in the histological composition of the marrow.

3. In marrows of normal adult rabbits, the principal variable affecting the oxygen consumption is the myeloid-erythroid ratio. When the proportion of myeloid cells increases, the rate of oxygen consumption is raised. This is at least partly due to the larger average size of the myeloid cells.

4. The relatively mature normoblast cannot be considered to be a metabolically inactive cell, for its rate of oxygen consumption, in proportion to its size, is not markedly different from that of the average myeloid cell.

5. The immature erythroid cell exhibits a relatively high rate of oxygen consumption.

6. In the myeloid series, the age of the cells is of less importance than in the case of the erythroid series, but intense granulation is probably associated with a high rate of oxygen consumption.

7. In extreme cases, a high percentage of fat in the marrow is associated with a low rate of oxygen consumption and vice versa, but in general, the fat content of the marrow is not a good index of its metabolic activity.

8. In the cases of two abnormal animals, the changes in the marrow, both cytologically and metabolically, were of such magnitude as to be readily detectable by the methods described.

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ELECTROLYTES IN NERVE

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There is much information available in the literature concerning the histological structure of nerve and the electrical and metabolic changes resulting from nervous activity. Modifications in the function of the nerve resulting from changes in the electrolytes in the environment have also been widely investigated. With the exception however of some microchemical-histological observations, which are not always easy to interpret, there has been no serious attempt to investigate the physico-chemical organization of the nerve. We have been unable to find in the literature any figures even for the normal content of the much-studied frog nerves in potassium, sodium, calcium, and chloride. This study was begun in the hope of discovering just what electrolytes are present in nerve, in which part of the nerve each substance is present and what the laws are which determine how these electrolytes exchange between the nerve and the environment. Some of our results were reported at a sypmosium in Chicago, June, 1933 and have since been published (Fenn, 1934). In this paper we propose to describe further experiments and to analyze the data as a whole more carefully than was formerly possible.

The general plan of the experiments consisted in analyzing frog nerves for potassium, sodium, chloride, and bicarbonate before and after immersion in Ringer's solution modified in various ways. For the present we have not been much concerned with the question whether the nerve retained its irritability or not or whether the solutions were "physiological" or not but have simply tried to determine, under various conditions, what electrolytes were present, in what quantities, and in what part of the nerve.

METHODS. All the experiments were concerned with frog nerve (*R. pipiens*) with the exception of a few experiments on crab and lobster nerve.

Potassium. Three or more nerves were necessary for an analysis. The method was the same as that used for muscle (Fenn and Cobb, 1934). It consisted in general of dry ashing the nerves overnight in a platinum crucible at about 600°C. and analyzing the ash by the platinic chloride method of Shohl and Bennett (1928).

Chloride. We are much indebted to Prof. A. N. Richards for communicating to us the details of the method developed in his laboratory by Doctor Westfall for micro-

analysis of glomerular urine. It has given satisfactory results in our hands and is sufficiently sensitive to permit analysis of the chloride content of a single frog sciatic nerve. For these analyses the nerve was introduced into a test tube, rubbed to a paste against the side with a glass rod in 4 cc. of water, allowed to stand 60 minutes, centrifuged and decanted. A few milligrams of Ag_2CrO_4 plus a crystal of $ZnSO_4$ were then added in the cold room and allowed to stand 1 minute with stirring. The suspension was then filtered through a no. 5 Whatman filter paper (no. 40 apparently allowed some Ag_2CrO_4 to pass through). Aliquots of this filtrate (0.3 cc.) were then added to 5 cc. of 95 per cent ethyl alcohol plus 4.2 cc. of diphenyl-carbazide reagent (0.5 gm. dry plus 70 cc. 95 per cent ethyl alcohol plus 25 cc. glacial acetic acid made up to 100 cc.). The resulting color was matched in a colorimeter against a known NaCl standard treated in the same way. The standard was made to match the unknown fairly closely on account of a small amount of Ag_2CrO_4 which dissolves even in the absence of chloride. For chloride analyses in blood plasma we used Patterson's modification of the open Carius method as described by Peters and Van Slyke (1932).

Bicarbonate. In many cases the solutions were equilibrated with 5 per cent CO_2 plus 95 per cent O_2 with $NaHCO_3$ added to give the desired pH. This offers the advantage that a determination of the HCO_3 content of the nerve permits an estimate of the pH of the nerve by an application of the Henderson-Hasselbalch equation. The limitations of this method are fully appreciated (Meyerhof, Möhle and Schulz, 1932; Brookens, 1933), and the results are interpreted accordingly. The bicarbonate content was determined by inserting a nerve in a differential volumeter in 1 cc. of unbuffered Ringer with 0.3 cc. of 25 per cent citric acid in a side arm. A 5 per cent CO_2 plus 95 per cent O_2 mixture is passed through the respirometer and after a period of equilibration the acid is tipped onto the nerve and the CO_2 evolved is measured. We have used a pK_1 value of 6.17. For further details see Fenn and Cobb (1934).

Sodium. Our first sodium analyses were carried out according to the method of McCance and Shipp (1931) and their modification for blood. In later experiments we used the modifications described by Salit (1932) which gives a more complete precipitation of the sodium (1.5 times as much precipitate for the same amount of sodium). About 35 mgm. or more of nerve or 0.217 cc. of plasma sufficed for an analysis. The method depends upon the precipitation of sodium as the triple acetate with uranium and zinc and its colorimetric determination in the dissolved precipitate with $K_4Fe(Cn)_6$. Nerves were first ashed in platinum crucibles in an electric oven overnight at 600°C. Control experiments showed that no sodium was lost in this process. Conversion of the salts to sulfates before ashing made no difference in the result. The precipitate was taken up in HCl and analyzed as usual. In using the method of McCance and Shipp for muscle it was found necessary to remove the potassium by precipitation with platinic chloride but the larger Na/K ratio in nerve made this unnecessary in the present experiments. It is unnecessary even in muscle if Salit's method is used. The method of McCance and Shipp gave large errors unless the colorimetric standard and the sample were equal in strength within 15 per cent. Otherwise the error was about 10 per cent. Analyses of salt samples before and after ashing in the furnace fell within the experimental error (about 5 per cent). For plasma analyses we have taken 0.217 cc. of plasma in a 10 cc. flask containing 3 cc. of water, added 0.3 cc. of 30 per cent trichloracetic acid, allowed it to stand 5 minutes, added water to 10 cc. mark, filtered, and analyzed for sodium in 2 cc. of the filtrate.

RESULTS. *Potassium.* The average potassium content of freshly

dissected nerves in March was found to be 4.8 ± 0.44 milli-equivalents per 100 grams fresh nerve. This was an average of six determinations. November frogs (7 analyses) gave a significantly lower value of 3.02 ± 0.25. This seems to be a true seasonal variation. In this respect autumn frogs are probably similar to summer frogs for De Boer (1918) has found that less potassium and more calcium is necessary in Ringer's solution to keep the hearts of summer frogs beating.

Nerves of November frogs were soaked for 5 hours in Ringer's solution of varying potassium content. At a critical potassium concentration of

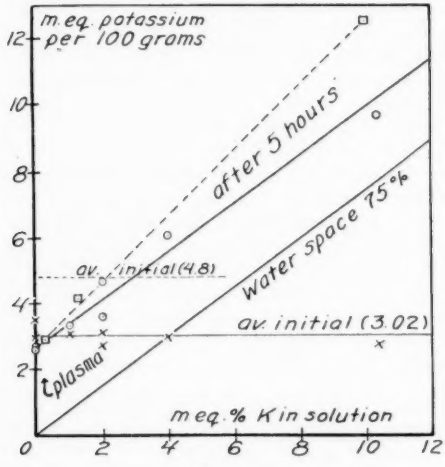

Fig. 1

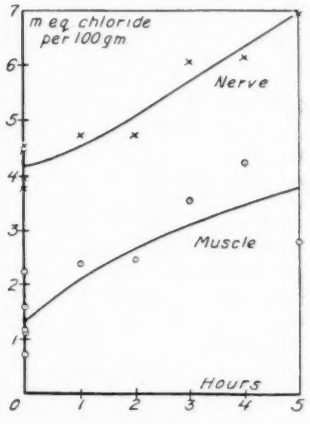

Fig. 2

Fig. 1. Potassium contents of nerves after soaking for 5 hours at 22°C. in solutions of different potassium concentration. Ordinates: milli-equivalents of potassium per 100 grams of soaked nerve. Broken lines, March frogs; solid lines, September and November frogs. See text.

Fig. 2. Increase in chloride content of nerve and muscle during immersion for various times in Ringer solution at 22°C. See text p. 83.

0.5 milli-equivalent per cent the potassium content of the nerves remained unchanged. This is near the potassium concentration of normal frog plasma which was found to be 0.25 milli-equivalent per cent. In solutions of higher content the nerves gain potassium. This behavior is illustrated in figure 1. The initial potassium content of unsoaked nerves is represented by crosses and the contents after 5 hours are represented by circles. To represent the soaked nerves a straight line has been drawn which has the same slope as the diagonal through the origin marked "water space, 75 per cent." This diagonal line would represent the potassium content of nerves if this substance were present in the nerve water in the same

concentration as in the outside solution. Since some 40 per cent of this nerve water is inside the fibres it is evident that some at least of the potassium which diffuses into a nerve from these concentrated solutions must go into the nerve fibres themselves. All of the increased potassium cannot be explained merely by an increase in concentration in the tissue spaces equal to that outside. Some potassium must therefore have diffused against the concentration gradient presumably by exchange with hydrogen ion. This is still more evident in the somewhat steeper slope of the dotted line (points indicated by squares) shown in figure 1 which represents an early experiment in which the nerves were soaked for 16 hours at 22°C. and figures for which have already been published (Fenn, 1934—table 2). In this case the initial potassium content of unsoaked nerves was 4.8 milli-equivalents per 100 grams instead of 3.02 and the critical potassium concentration below which the nerve loses potassium was about 2 milli-equivalents per cent.

TABLE 1

Potassium contents of nerves after soaking 16 to 18 hours at 4 to 5°C.

(Milli-equivalents per 100 grams of initial weight)

K IN SOLUTION	NUMBER OF ANALYSES	K IN NERVE	
		pH 6.3-7.7	pH 7.0
0	6	2.27	2.43
0.2	14	3.79	3.63
1.0	3	3.92	3.92

Average initial content, 4.8.

Further data on nerves of spring frogs are shown in table 1. Potassium concentrations of 0, 0.2, and 1.0 milli-equivalent per cent were used, in all of which potassium was lost by nerve, the initial content being 4.8 milli-equivalents per cent.

In these analyses the pH varied between 6.3 and 7.7 in different experiments. Since the pH did not seem to make any difference in the diffusion of potassium the results of all the analyses were averaged together. If, however, only the analyses at pH 7.0 are included the result is almost the same.

Effect of pH. By analogy with muscle (Fenn and Cobb, 1934) and on theoretical grounds it is to be expected that potassium will diffuse across a membrane from the more alkaline to the more acid medium more readily than in the reverse direction. This may be due to its diffusion as KOH (Osterhout, 1930) or to a compensatory diffusion of hydrogen ions in the opposite direction.

It has already been mentioned that the pH did not seem to affect the

diffusion of potassium in our experiments. This was tested more directly in four experiments in which paired nerves were soaked in solutions of 7.8 mgm. per cent potassium content, one set of nerves at pH 6.3 and the matched set at pH 7.7. After soaking for 16 hours at 22°C. the nerves were analyzed. The results of four experiments showed 116, 153, 147, and 187 mgm. per cent potassium at pH 6.3 as compared to 107, 152, 155 and 129 mgm. per cent at pH 7.7. Average values were 151 and 136 mgm. per cent at pH 6.3 and 7.7 respectively. This difference is not large enough to be significant in so small a series, and it is in the wrong direction to conform with the theoretical expectation. It is possible that the expected pH effect might be obtained if other solutions were chosen over another pH range. These solutions were equilibrated with 5 per cent CO_2 and 95 per cent O_2 except in the last of the four experiments in which the solution was equilibrated with O_2 only, and contained only phosphate buffers. To obtain a pH of 7.7 with 5 per cent CO_2 a large amount of $NaHCO_3$ is required and this necessitates a diminution in NaCl to maintain the osmotic pressure (see table 2).

The initial pH inside the nerves as calculated from the bicarbonate content was 7.15 in 5 per cent CO_2 plus 95 per cent O_2. After soaking for 16 and 19 hours at 22°C. at pH 6.3 this decreased in three experiments to 6.88, 6.70 and 6.81 respectively (av. 6.80) while after soaking at pH 7.7 the internal pH increased to 7.64, 7.51 and 7.63 (av. 7.59) in the paired nerves of the same frogs. This change in pH from 6.80 to 7.59 is equivalent to a change in average bicarbonate from 14 to 84 volumes per cent. These solutions were equilibrated with 5 per cent CO_2 plus 95 per cent O_2. When taken out of these solutions and put into a respirometer in oxygen without solution, the nerve from the pH 7.7 solution showed a rate of oxygen consumption which in two experiments was 18 and 100 per cent, respectively, greater than that of its mate. Two other matched nerves in phosphate buffers at pH 6.3 and 7.7, respectively, in oxygen showed 55 per cent greater rate of oxygen consumption in the alkaline solution. When subsequently equilibrated with 5 per cent CO_2 and 95 per cent O_2 these nerves gave carbon dioxide contents of 5.8 and 16.8 volumes per cent corresponding to an internal pH of 6.41 and 6.88 respectively (instead of 6.80 and 7.59), both being therefore more acid than the nerves soaked in bicarbonate buffers of the same pH. This is to be expected since the nerves equilibrated with CO_2 were more acid inside during the period of immersion and tended to retain base.

By analyses of the chloride, potassium and sodium in nerves in these same solutions at pH 6.3 and 7.7 we have obtained some evidence to show how these changes in internal pH are brought about. Many nerves were obviously necessary for these analyses. Each figure is the average of several experiments. The data are recorded in table 2.

Both solutions contained 12.3 milli-equivalents of sodium and 0.2 milli-equivalent of potassium per 100 cc. of solution (calcium was omitted to avoid precipitation). The difference between them consisted in replacing some NaCl by an equivalent amount of $NaHCO_3$. After soaking in these solutions some nerves were analyzed for sodium, some for potassium, etc., and the results are tabulated. The differences between the acid and alkaline nerves in their electrolyte contents are found to correspond to the differences between the two solutions, for their sodium and potassium contents are nearly equal while the alkaline nerve is found to contain less chloride than the acid nerve and correspondingly more bicarbonate. As far as the differences between the two nerves are concerned, therefore, the electrolyte balance is fairly complete. The information regarding the

TABLE 2

Differences in electrolyte content of matched nerves soaked in Ringer at pH 6.3 and 7.7 respectively

(Milli-equivalents per 100 grams initial weight)

SOLUTION			NERVE AFTER SOAKING				
pH	Cl	HCO_3	Cl	HCO_3	K	Na	pH
6.3	11.3	0.2	7.30	0.65	2.97	8.95	6.8
7.7	4.2	5.35	4.17	4.17	2.74	9.00	7.6
Differences			−3.13	3.52	−0.23	0.05	
Average initial values			4.6	1.11	4.83	5.6	7.15

pH	GAIN IN M.-EQ. PER 100 GRAMS NERVE	
6.3	1.49 cations	3.16 anions
7.7	1.31 cations	2.02 anions

individual nerves, however, is not complete as can be seen by comparing the electrolyte contents after soaking with the average initial contents of similar nerves. In the acid nerve, for example, it is found that during soaking, potassium is lost while bicarbonate, chloride and sodium are gained, there being a net gain of 1.49 milli-equivalent of cations and 3.16 milli-equivalents of anions. Some other anion must have escaped from the nerve—perhaps lactate or phosphate—or some other cation must have entered—partly hydrogen ions. In the alkaline nerve a better check was obtained with a net gain of 1.31 milli-equivalents of cations and of 2.02 milli-equivalents of anions.[1]

Sodium and chloride. The experiment just recorded shows that the chloride and bicarbonate anions readily exchange between the inside and

[1] The change in weight of these nerves after soaking was not over 10 per cent, usually a gain. Nerves from slightly edematous frogs generally lose weight.

the outside of the nerve. If we assume that the nerve fibre is impermeable to anions it must be concluded that the chloride at least is not located in the fibres, but in the tissue spaces or connective tissue. This we believe to be true and many other experiments have demonstrated the ease with which chloride can exchange with other anions. Thus paired nerves were soaked for 5 hours, one in 0.105 M NaCl and the other in 0.105 M $NaNO_3$. Both solutions contained M/150 phosphate buffers at pH 7.25. After soaking, one nerve contained 209 mgm. per cent chloride and the other only 39 mgm. per cent. A similar experiment showed 103 mgm. per cent in a NaCl solution and only 25 mgm. per cent in a Na_2SO_4 solution.

These were preliminary experiments in which analyses were made by a nephelometric method using $AgNO_3$ in a HNO_3 extract of the nerves. More extensive measurements were made with the Westfall chromate

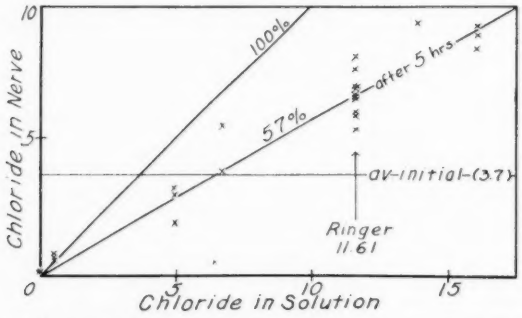

Fig. 3. Chloride content of nerve after soaking for 5 hours at 22°C. in Ringer's solutions of varying chloride content, NaCl being replaced by $NaNO_3$. Ordinates: milli-equivalents chloride per 100 grams of soaked nerve. Abscissae: milli-equivalents of chloride per 100 cc. of solution.

method which gave more reliable results. Many of the figures so obtained are recorded in figure 3 in which the chloride content of the nerve is plotted against the chloride concentration in the solution. In order to vary the chloride content the NaCl was replaced in varying amounts by $NaNO_3$ or was made slightly hypertonic by addition of NaCl. The solution contained in addition 0.02 per cent $CaCl_2$, 0.01 per cent KCl, and M/150 phosphate buffers at pH 7.25. The nerves were soaked for 5 hours at 22°C. It is seen that as the chloride content of the solution decreases the chloride content of the nerves falls along a diagonal through the origin of such a slope that the concentration inside (per 100 gram of nerve) is always 57 per cent of that outside. Since there is this close proportionality between the inside and the outside concentration it is probable that 57 per cent of the nerve (after soaking) contains chloride in the same concentration as it exists in plasma.

In another similar series of experiments the sodium contents of nerves were determined after soaking for 5 hours in Ringer's solution with varying sodium content. The sodium content of the solution was varied by the addition of NaCl or by substituting isotonic glucose for NaCl, leaving KCl, $CaCl_2$ and phosphate buffers unchanged. The results are plotted in figure 4. A straight diagonal line has been passed through the origin of the same slope as that which represented the chloride points in figure 3, i.e., all points along this line represent nerves containing 57 per cent as much sodium per 100 grams (weighed after immersion in the solution) as exists in the solution. The experimental points lie slightly above this line in the low concentrations of sodium as if the sodium did not diffuse out as easily as the chloride, but on the whole the agreement is fairly good.

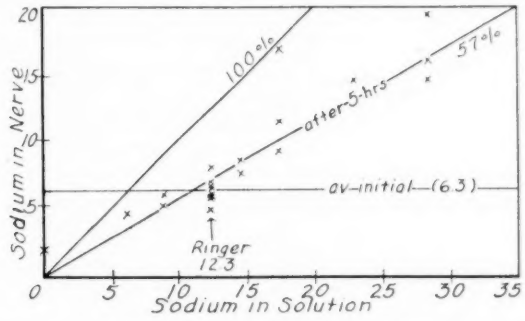

Fig. 4. Sodium content of nerve after soaking 5 hours at 22°C. in Ringer's solution of varying sodium content, NaCl being replaced by an osmotic equivalent of dextrose. Concentrations as in figure 2.

From these experiments it may be concluded that 57 per cent of the weight of the nerve after soaking in Ringer's solution is "sodium chloride space" and contains NaCl in concentrations identical with those obtaining in the solution.

Further experiments were undertaken to ascertain whether this was also true of the nerve in situ in the body. The experiments consisted of making analyses for sodium and chloride in the blood plasma of the frog and in the nerves immediately after dissection and without exposure to Ringer's solution.

The blood for these experiments was taken with a syringe direct from the aorta. Three other methods were tried: snipping off the whole head and catching the blood in a funnel; snipping off the end of the ventricle over a tube; and bleeding from the abdominal aorta which is held over a tube from an opening in the back of the frog as described by Mond and Netter (1932). In the last method it seemed that tissue juices often ran down the outside of the vessel into the centrifuge tube and low hematocrit values resulted. A similar objection applied to both the other

methods. The syringe method will also give low hematocrit values if the drawing of the blood is continued for a sufficient length of time since fluid is drawn into the depleted circulation from the tissues. The syringe contained about 1 mgm. of dry heparin and the blood was always oxygenated before being centrifuged. The resulting loss of CO_2 was probably responsible for the slight precipitation of calcium which usually was observed as a tiny white button about 1 mm. in diameter in the bottom of the centrifuge tube. This material was finely crystalline under the microscope and gave the oxalate test for calcium. A 0.217 cc. sample in a blood-counting pipette sufficed for each analysis of sodium or chloride. In each experiment (of table 3) the frog was a good sized female kept in running water at room temperature in February.

The results of four such experiments are collected in table 3. The expected sodium content of the nerve has been calculated on the assumption that it should bear the same ratio to the chloride as it does in the blood

TABLE 3

Sodium and chloride in nerve and plasma of frogs

(M.-eq. per 100 cc. plasma or per 100 grams nerve)

PLASMA		NERVE		CALCULATED	
Na_p	Cl_p	Na_n	Cl_n	Na_n	Excess Na
10.48	7.94	6.66	4.18	5.52	1.14
10.00	7.60	5.89	3.60	4.74	1.15
11.10	6.56	6.60	2.73	4.62	1.88
9.94	7.64	7.01	4.41	5.74	1.27
Av.....10.38	7.43	6.53	3.73	5.16	1.36
Na/Cl.. 1.4		1.75		1.4	

Av. "Na space" $\frac{6.53}{10.38} = 63\%$; Av. "Cl space" $\frac{3.73}{7.43} = 50\%$.

plasma. There is found to be an average excess of sodium in the nerve of 1.36 milli-equivalents per 100 grams of nerve. In other words, the ratio Na/Cl is 1.75 instead of 1.4 as in the plasma. Further, the sodium space is 63 per cent and the chloride space 50 per cent of the weight of the nerve, while in soaked nerves both are 57 per cent. The difference between 63 and 57 per cent is probably explained by the approximate 10 per cent increase in weight of the nerve after soaking since the latter was calculated on the basis of the weight after swelling. These experiments, therefore, indicate that on soaking a nerve in Ringer's solution, there is a greater intake of chloride than of sodium, the Na/Cl ratio increases and the excess sodium largely disappears.

This large intake of chloride by soaked nerves was noted previously (Fenn, 1934). It is partly explained very simply by the fact that the

Na/Cl ratio in the Ringer's solution is near unity (1.05) while in frog plasma it may be as high as 1.85 although the average value in table 3 is 1.4. Likewise the average value in 25 other frogs was 1.45. The very high values seem to be characteristic of hibernating frogs, or frogs in poor condition, where the chloride in extreme cases may be as low as 4.0 milli-equivalents per cent. The sodium content is also lower than normal in such frogs but not often lower than 9.0 milli-equivalents per cent.

The sodium and chloride contents of nerves were previously reported (Fenn, 1934) as 5.6 and 4.6 milli-equivalents per cent respectively in spring frogs. Later analyses in June and July gave values of 6.38 milli-equivalents per cent for sodium in 14 analyses and a single value of 3.75 for chloride. In September and October the average sodium content found in 13 analyses was 6.35, and 4 analyses for chloride in February gave an average value of 3.45 milli-equivalents per cent. These analyses serve to confirm the figures found in table 3 of 6.5 and 3.7 milli-equivalents per cent for sodium and chloride respectively. Possibly the earlier values were due to lack of technical refinements in the analyses rather than to a seasonal difference in the frogs. It is concluded, therefore, that in the nerves a larger fraction of the sodium is not combined with chloride than in the plasma. This is true also in muscle and it is to be expected in an environment where the protein content is higher than in plasma. Possibly also this excess sodium is located not outside the fibres but inside in the potassium space.

The increase in chloride content described for nerve after soaking in Ringer's solution is found also in muscle. Data illustrating this increase are plotted in figure 2 as a function of time for both nerves and muscles. Since each point represents an individual nerve or muscle the exact shape of the curve is not very reliable. Probably both nerve and muscle attain a maximum equilibrium chloride content after 5 hours. While the high chloride content of the Ringer compared to the plasma is largely responsible for this increase in anion content, no explanation is thereby offered of the mechanism involved (i.e., what compensatory changes occur in other ions). In nerves the bicarbonate content does not change materially during this time and the only other significant change in electrolytes is a loss of potassium of about 1 milli-equivalent per cent, which, however, is in the wrong direction to explain the access of chloride. A loss of phosphoric, lactic or other organic acids or a formation of ammonia would explain the result. As illustrated in figures 3 and 4 the nerves gain chloride but in general not sodium when soaked for 5 hours in Ringer's solution. Both sodium and potassium, however, have not been measured on the same nerves. It is probable that nerves which gain sodium in Ringer also lose potassium while those that do not lose potassium (autumn frogs) do not gain sodium.

Isotonic sugar solutions. The view that potassium is inside and sodium chloride outside the fibres is supported by observations on the loss of these salts from the nerve during immersion in isotonic sugar solution. The results of a number of analyses are plotted in figure 5. The graphs show the concentration of sodium, chloride, and potassium in milli-equivalents per 100 grams of initial weight of nerve at various times after immersion in the sugar solution. It is obvious that sodium and chloride are lost fairly rapidly in about equal amounts while the potassium leaks out more slowly, 60 per cent of the initial content remaining inside the nerve for 12 hours. The last of the sodium apparently diffuses out less readily than the chloride, being combined presumably with indiffusible anions or confined inside the fibres (cf. excess sodium, table 3). Some more recent experiments indicate that the chloride comes out much more rapidly than

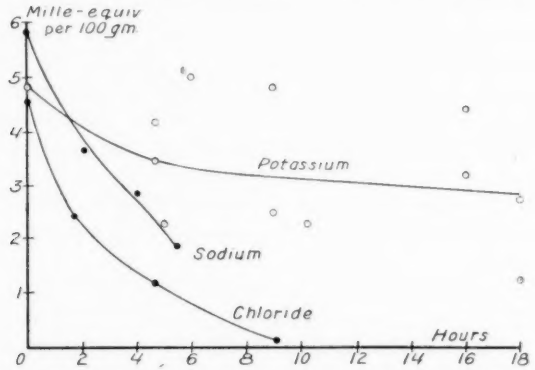

Fig. 5. Decreasing concentration (ordinates) of potassium, sodium and chloride in frog nerves soaked for varying times in isotonic dextrose solution (4.5 per cent).

is indicated on this graph, so that there is scarcely any chloride left after 1 hour. Whatever may be the cause of this difference it seems apparent that in general sodium chloride readily escapes from the nerve into sugar while potassium is retained, probably by the indiffusibility of its anions. In this respect the nerve behaves very much like muscle, where the same conclusion was drawn, i.e., that sodium and chloride are largely present in the tissue spaces.

Lobster and crab nerves. During the summer of 1933 at Woods Hole, one of us (A. H. H.) collected some data on electrolytes in the spider crab (*Libinius marginata*) and lobster (*Homarus americanus*) nerves. Analyses for sodium and potassium were made at the Rochester laboratory. Nerves for this purpose were dried in small tubes and shipped by mail.

The initial contents of these nerves in sodium, potassium, calcium, and chloride are given in table 4.

suming the same to be true for the species which we used, the data given by Bethe and Berger for sea water have been included in table 4 for comparison with the data for nerves.

The significant points in the table are as follows: 1. Potassium is 12 to 15 times as concentrated inside as outside even if it is assumed to be distributed equally throughout the whole substance of the nerve. It must be still more concentrated in the water within the nerve fibres themselves. 2. Sodium slightly exceeds the chloride in concentration in crab nerve as it does in frog nerve and muscle and plasma. 3. The "sodium chloride space" represents 25–32 per cent of the weight of the nerve (i.e., much less than in medullated frog nerve), assuming that sodium and chloride are present in this space in the same concentration as in sea water. 4. In

TABLE 4

Comparison of electrolyte content of sea water and nerves

(Milli-equivalents per 100 grams)

	SEA WATER	CRAB NERVES	LOBSTER NERVES
Na	53.0	16.8	—
K	1.3	15.8	20.3
Ca	2.6	13.5	13.0
Mg	10.1	—	—
Cl	54.7	13.4	10.7
P	—	2.5	—

crab nerve our analyses account for only 61 millimols per 100 grams water in the nerve as compared to 115 in the sea water. Some other ions must be present in considerable amounts to equalize the osmotic pressure inside and outside. 5. The analyses in crab nerves show 46.1 milli-equivalents as cations and only 15.9 milli-equivalents as anions. The missing ions are therefore chiefly anions. 6. Calcium is at least five times as concentrated in nerve as in sea water, and is nearly as abundant as potassium. 7. Mg must be approximately as concentrated in nerve as in sea water if the total concentration of cations in the nerve water is equal to that in the sea water.

Some experiments were also performed in which the nerves were soaked in artificial sea water containing varying amounts of potassium and equilibrated with approximately 4 per cent CO_2 plus 96 per cent O_2. As the potassium concentration was increased the NaCl was decreased in equivalent amounts. The highest concentration was reached in "KCl sea water" in which all the NaCl was replaced by KCl. Most of the solutions used

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On pages 85 and 89 of Volume 110, due to an error by the senior author, the figures quoted for calcium in the last two columns of table 4 and the third column of table 5 are 10 times too high.

According to the data of Bethe and Berger (1931) the electrolyte contents of the body fluids of the European lobster (*Homarus vulgaris*) and the green crab (*Carcinus maenas*) do not differ much from that of sea water. Assuming the same to be true for the species which we used, the data given by Bethe and Berger for sea water have been included in table 4 for comparison with the data for nerves.

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were far outside of the physiological range. They were used only as a preliminary experiment with the expectation of continuing later with more physiological solutions. The results are presented in figures 6 to 9.

The dotted diagonal (fig. 6) shows the graph which would be expected if the amount of potassium per 100 grams nerve (after soaking) was always exactly equal to the amount of potassium per 100 cc. of solution. In most of the solutions the nerves contain more potassium than would be expected on this basis. This is of course especially true in the dilute solutions.

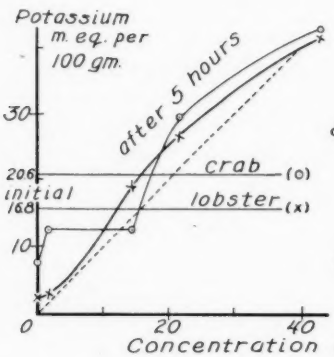

Fig. 6

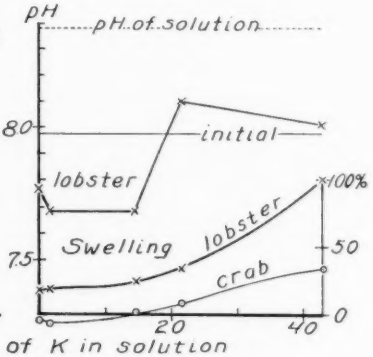

Fig. 7

Fig. 6. Potassium contents (ordinates) of crab and lobster nerves after 5 hours' immersion at 20°C. in sea water and increasing potassium content (abscissae). Concentrations are expressed in milli-equivalents per 100 grams of nerve (weighed after immersion) or per 100 cc. of solution. Each point is the average of at least two determinations on separate nerves.

Fig. 7. Lower curves show the gain in weight of crab and lobster nerves in per cent of the initial weight after soaking for 5 hours in sea water of increasing potassium content (abscissae). Each point is the average of at least three, usually seven or more, determinations. Upper curves show the calculated pH inside the nerves after soaking. The solubility of CO_2 in the nerve was taken to be 0.77 cc. per gram of nerve per atmosphere pressure which is the same as the solubility of CO_2 in the water of the nerve (87.8 per cent H_2O).

The difference would be even greater if allowance were made for the water content of the nerves (84 to 88 per cent—Gerard, 1932). Crab nerves endure exposure to artificial sea water better than lobster nerves. This is indicated by the fact that crab nerves tend to retain their potassium better than lobster nerves when exposed to potassium-free sea water or artificial normal sea water. Cowan (cf. Hill, 1932 and 1934) has also observed a loss of potassium from crab nerves accompanied by a loss of irritability. In high concentrations of potassium both nerves show potassium contents which do not differ much from that of the solution. This is true in lobster

nerves, even after soaking for 5 hours in artificial sea water which seems to be a very injurious treatment for them. At concentrations above 15 to 20 milli-equivalents per 100 grams of solution the nerves gain potassium, in lower concentrations they lose potassium. There is no good evidence in these experiments of a movement of potassium against the concentration gradient.

Further evidence that crab nerves survive better than lobster nerves in artificial sea water is shown in figure 8 which represents the swelling in per cent of the initial weight of both lobster and crab nerves in solutions of different potassium content. Evidently lobster nerves swell more than crab nerves for the same solution. In both nerves the swelling is greater in solutions of high potassium content. This is possibly due to an injury to the nerve which permits anions to penetrate along with the cations. The osmotic pressure inside and out would then always be equal except for the unopposed colloid osmotic pressure of the nerve which causes continuous swelling.

Some determinations of the bicarbonate content of lobster nerves were made after they were soaked in the same KCl-sea water solutions under known carbon dioxide tensions. This permitted a crude calculation of the pH inside the nerve. The results are plotted in the upper part of figure 8. The calculated pH of the solution was 8.37 which is considerably more alkaline than the initial pH inside the nerves. In spite of the greater alkalinity of the solution the nerves become more acid inside after soaking 5 hours in solutions of low potassium content. In general the curve indicates that where potassium is gained the pH increases and vice versa.

The sodium contents of crab nerves after soaking for 5 hours in these same solutions of different potassium contents are shown in figure 8. The diagonal dotted line indicates the sodium content of the solution which was decreased in proportion to the increase in potassium. The results show that in normal or potassium-free sea water there is an increase in the sodium content of the nerve. This gain in sodium is correlated with a loss of potassium in these same solutions as shown in figure 6. This may be interpreted as meaning that soaking the nerve causes an increase in the size of the sodium space. In the crab nerves, at least, this increase must occur at the expense of the fibres, which liberate their potassium, for there is no swelling of the nerve as a whole in these low potassium solutions. As the concentration of potassium is further increased the concentration of sodium in the sodium space decreased in rough proportionality with the decrease in sodium in the solution.

We are indebted to Dr. S. R. Tipton for some analyses of calcium in lobster nerves after soaking in these same solutions. Here also calcium, like sodium, is gained in potassium-free or artificial sea water, but is lost in solutions of higher potassium content (see table 5).

Figure 9 shows similar data for the chloride content of both lobster and crab nerves after soaking for 5 hours in the same solutions. The chloride always tends to diffuse from the more concentrated to the less concentrated solution with the result that the low initial chloride content of the nerve tends to approach the much higher content of the solution, which is constant throughout the series. Gain in chloride is greatest in the very low or very high potassium concentrations, leaving a minimum at 15 to 20 milli-equivalents per cent potassium which is not well explained.

Analyses for other important inorganic constituents of the nerve are required before the complete electrolyte balance can be determined with

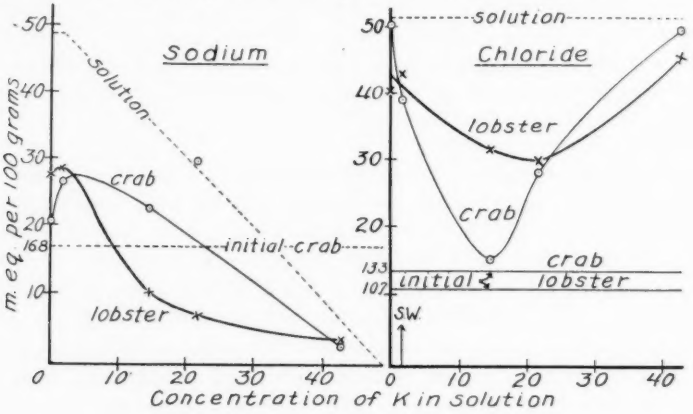

Fig. 8 Fig. 9

Fig. 8. Sodium content of nerves after soaking for 5 hours. Expressed in milli-equivalents per gram of nerve as weighed after soaking. The initial sodium content of lobster nerve is lacking.

Fig. 9. Chloride content of nerves after soaking 5 hours expressed in milli-equivalents per gram of nerve as weighed after soaking.

certainty. It is profitable however to consider the indications which are obtained from the sodium, potassium, chloride, calcium, and bicarbonate (pH) data. These are summarized in table 5, where the changes in the content of these constituents and of water due to the soaking are tabulated. The change in water content as well as the initial electrolyte contents are also included. In general the figures are all averages of two or more analyses.

In the last column of table 5 are given the unexplained movements in terms of chloride. It is obvious that both in potassium-free sea water and in KCl-sea water, there is a considerable intake of chloride which is not balanced by an intake of sodium or potassium. In fact, in potassium-

free solutions potassium goes out from the nerve in spite of the large intake of chloride while in KCl-sea water sodium goes out in spite of the large influx of chloride. There is no very certain correlation between the pH changes and the general electrolyte balance although there is some indication that the greatest intake of unbalanced chloride was observed in low-potassium solutions in which the nerves become acid. It would appear that this large intake of chloride must be balanced by a loss of some other

TABLE 5

Gain (+) or loss (−) in electrolyte concentration of crab and lobster nerves after soaking 5 hours

(Milli-equivalents per 100 grams initial weight)

CONCENTRATION OF K IN SOLUTION	WATER, CC. PER 100 GM.	Ca	Na	K	Cl	HCO_3	Cl UNEXPLAINED
			Crab				
0	−4	—	+2.8	−8.9	+34.8	−2.0	+38.9
1.45	−8	—	+7.8	−4.4	+22.6	−3.3	+15.9
14.5	+0.7	—	+5.4	−3.6	+1.7	−3.6	−3.7
21.7	+8	—	—	+15.9	+16.9	+3.2	—
42.9	+31	—	−13.0	+40.4	+51.7	+2.6	+26.9
Initial content.....		13.5	16.8	15.9	13.3	8.2	
			Lobster				
0	+18	+5.4	+15.8	−17.9	+36.6	—	+33.3
1.45	+19	+8.3	+17.0	−17.4	+40.4	—	+32.5
14.5	+26	−2.4	−4.2	+3.2	+28.9	—	+32.3
21.7	+34	−3.4	−7.9	+14.9	+28.9	—	+25.3
42.9	+100	−1.2	−10.4	+63.0	+80.3	—	+28.9
Initial content.....		13.0	16.8*	20.6	10.7	—	

* For purposes of calculation the initial sodium content of lobster nerve is assumed to be equal to that found in the crab nerves.

Bicarbonate data are lacking for the lobster and calcium data for the crab. We are much indebted to Dr. S. R. Tipton for the calcium analyses.

anion from the nerve, since it is hardly possible to suppose that magnesium, the only undetermined cation, could play so large a rôle. Other evidence for the presence of some other undetermined anion in the crab nerves has already been presented (table 4). Some of the changes in electrolyte content can be explained by the change in water content but in general the electrolyte changes far exceed the change in water. Thus in crab nerves the maximum swelling in KCl-sea water was only 31 per cent, whereas the chloride content was increased nearly 400 per cent.

A few measurements were made of the inorganic and phosphagen phosphorus in crab nerve. These indicate a decrease in the inorganic fraction from 23.5 to 12 or 13 mgm. per cent phosphorus after soaking for 5 hours either in high or low concentrations of potassium (0 to 14.5 m.-eq. per cent).

TABLE 6

Probable electrolyte distribution in frog nerve

(Millimols per 100 grams nerve or 100 cc. plasma)

	NERVE	PLASMA	NERVE	
			NaCl-space	K-space
Na	6.20 (16)	10.38	5.19	1.01
K	4.80 (6)	0.25 (2)	0.13	4.67
NH_4	0.19	—	—	0.19
Ca	0.36	0.20	0.10	0.26
Mg	0.80 (1)	0.30 (1)	0.15	0.65
Cl	3.70	7.43	3.7	0
PO_4	1.0	0.31 (2)	0.15	0.85
Lactate	0.80	0.80	0.40	0.40
HCO_3	1.08 (6)	2.54 (2)	1.27	0
S	0.35	—	—	0.35
H_2O	75	96 (1)	48	27
Millimols per 100 cc. H_2O	25.7	23.2	23.2	31.0
Cation equivalents	13.51	11.63	5.81	7.69
Anion equivalents	7.93	11.39	5.69	2.45

Figures for lactate in nerve were taken from data of Holmes, Gerard and Solomon (1930), and the concentration of lactate in plasma was assumed to be equal to that in nerve. Phosphorus content in nerve is based on data of Gerard and Wallen (1929). Phosphorus content of plasma is based on two analyses for total phosphorus which is taken to be divalent. The inorganic phosphorus, according to Walker (1933) is only 0.117 millimol per 100 cc. Bicarbonate contents of nerve and plasma were made in equilibrium with a mixture of 5 per cent CO_2 and 95 per cent O_2. Plasma analyses were made on the Van Slyke apparatus. Ammonium and water contents of nerve were taken from Gerard's summary (Gerard, 1932). Calcium analyses were made by Dr. S. R. Tipton, and have been published elsewhere (1934). The figure given for sulfur is one-third of the average total sulfur content of nerve as found by Hayasi (1933) in the *Finwal* and the *Seiwal*. This represents the lipid fraction (Tabulae Biologicae, III, 537) and may be largely a potassium cerebroside sulfate as described by Blix (1933). In this compound one potassium atom combines for each atom of sulfur. We are indebted to Dr. R. G. Sinclair for information concerning this compound. Where only a few analyses were performed the figures in parentheses indicate the number.

The variations in the phosphagen fraction were probably not significant (14.3 to 19.6 mgm. per cent P). It is at least evident that the phosphorus fractions do not account for many combining equivalents in the total electrolyte balance, since the initial phosphagen plus inorganic phosphorus

is only 37.8 mgm. per cent or at most 2.5 milli-equivalents per cent (assuming that P is bivalent).

Discussion. The foregoing experiments afford evidence in favor of the view that electrolytes in nerve may be considered as divided between a "potassium space" probably inside the fibres, and a "sodium chloride space" probably outside the fibres. It is very likely that this arbitrary division of the nerve into two spaces represents an undue simplification of the actual situation and some difficulties arise when one attempts to draw up a tentative distribution between these two spaces of all the electrolytes known to be present. Such an attempt is nevertheless instructive and the results of one such trial for frog nerve are included in table 6.

The first two columns of table 6 give the contents of nerve and plasma in various electrolytes expressed in millimols per 100 grams wet weight of nerve or per 100 cc. of plasma. The total amounts of the various electrolytes in nerve are approximately as previously published (Fenn, 1934) with a few minor changes. The last two columns show the amounts of each of these electrolytes which are believed to be contained in the potassium space and the sodium chloride space, respectively. In making this division it is assumed that 48 of the 75 cc. of water in the nerve is outside the fibres and contains electrolytes in the same concentrations in which they exist in plasma. Since plasma is 96 per cent water, exactly one-half of the electrolytes contained in 96 cc. of plasma water would be found in 48 cc. of the nerve water. This 48 cc. is arbitrarily chosen so as to contain all the chloride in the same concentration as it is found in the plasma. This also includes all but about one-sixth of the sodium. The rest of the nerve electrolytes which are not accounted for by the sodium chloride space are assigned to the potassium space in the amounts given in the last column (obtained by subtracting column 3 from column 1). This potassium space contains, therefore, the major fraction of the potassium together with a large fraction of the calcium, magnesium and phosphate. It is a striking fact that after making the bicarbonate concentration in the sodium chloride space equal to that in the plasma, there is no bicarbonate left over for the inside of the fibres (actually a negative amount). This presumably indicates a considerable degree of acidity in the potassium space. It depends upon the undoubted fact that the plasma bicarbonate is at least twice as high as the nerve bicarbonate. This confirms also the high value for plasma bicarbonate found by Wastl and Seliskar (1925). Such an acidity inside the fibres is of course to be expected in a Donnan membrane equilibrium where potassium is high inside and the membrane is permeable to potassium and hydrogen.

This high concentration of bicarbonate in the plasma compared to that in the nerve with which it is presumably in equilibrium requires some further comment, especially in view of a previous statement of ours (Fenn,

1934) that nerve neither loses nor gains CO_2 when immersed in a solution of approximately the same bicarbonate content or pH.[2] This earlier statement was based upon experiments on nerves after immersion for 5 hours in Ringer's solution. During this treatment the chloride space increases from 50 per cent to 57 per cent. Likewise the "bicarbonate space" increases from about 50 per cent to 75 or 100 per cent. A similar change apparently occurs in muscle which contains about 25 volumes per cent CO_2 compared to 57 volumes per cent in plasma when both are equilibrated with 5 per cent CO_2. Meyerhof, Möhle and Schulz (1932) found 1.37 times as much CO_2 in a bicarbonate solution or serum in equilibrium with muscle as in the muscle water, while Brookens (1933) found only 1.2 times as much after soaking for 3 to 8 hours. In both nerve and muscle, therefore, immersion in Ringer's makes the bicarbonate concentration more nearly equal inside and outside, as if the pH inside the fibres is increasing relative to that outside during immersion. A possible alternative to this conclusion is that nerves immediately after dissection and before immersion do not represent fairly the nerves in situ having already lost some combined CO_2. In this case more bicarbonate should be added to the nerve anions in table 6.

After total concentrations in millimols per 100 grams of water are calculated for the nerve spaces and for plasma, by addition of the several items of table 6, it is found that the total concentration in nerve is 25.7 as compared to 23.2 millimols per 100 cc. of plasma. The latter figure agrees well with the figure, 23.9, given by Hill and Kupalov (1930) for isotonic sodium chloride from vapor pressure measurements on frog muscle. The concentration in the sodium chloride space is of course made equal arbitrarily to that in the plasma, but the concentration in the potassium space reaches the high value of 31. This would indicate that the activities of some of these electrolytes are somewhat less in the nerve than in plasma.

An important fact is found when cation and anion equivalents in these spaces are compared, as in the last two lines of table 6. In plasma and in the sodium chloride space of nerves the cations are only slightly in excess of anions, the difference being probably due to proteins. In whole nerve and particularly in the potassium space, however, the cations are in larger excess over the anions, so that only 2.45 anion equivalents are available to combine with 7.69 cation equivalents. This would indicate that a considerable fraction of the potassium in the fibres is combined with some large indiffusible organic anion. We have found no way of avoiding this

[2] We have more recently confirmed it by measuring directly on a Van Slyke apparatus the CO_2 content of the solution used and finding it 24 volume per cent like the nerve, and, secondly, by tipping Ringer's solution containing 24 volume per cent CO_2 on to nerves in a differential volumeter in 5 per cent CO_2 and finding no absorption or evolution of CO_2 in consequence.

conclusion unless the chloride is assumed to be evenly distributed between the inside and the outside of the fibres.

According to the tentative scheme proposed in table 6, about one sixth of the sodium of nerve is contained within the fibres or in the potassium space within a membrane permeable to potassium but impermeable to sodium. Confirmation of this suggestion is found in the fact that approximately this amount of sodium seems to remain inside the nerve when immersed in isotonic sugar solutions (fig. 5). Likewise in table 3 it is shown that about one fifth of the sodium is in excess of the chloride ("excess Na").

We are well aware that the proposed scheme represents only one possible interpretation of our results. It is in conflict furthermore with observations by microchemical methods according to which (Macallum, 1905; Macallum and Menten, 1906; Macdonald, 1907) the chloride is contained chiefly inside the fibres while the potassium (according to Macallum, 1933) is contained in the surfaces of the axones. Macdonald (1905), however, using similar methods located potassium inside the axones. It is certainly improbable that such a large fraction of the total cations of the nerve as is represented by the potassium should be confined to the surfaces only. It is also not likely that the methods used could avoid the possibility that potassium diffused from the middle of the axones to the surface during precipitation. The finding of these authors that chloride is present only in the axones is more difficult to explain but it is equally difficult to believe that there is no chloride (or much less chloride) outside the fibres in the lymph and tissue spaces.

It may also seem improbable that so much of the nerve as 50 per cent is represented by "tissue spaces." This term may include of course connective tissue cells as well as the epineurium, perineurium, and blood vessels, or even part of the myelin sheaths. According to the conductivity measurements of Netter (1927) the tissue spaces represent 30 per cent of the nerve while according to the microscopical measurements of Ellison (1910) the axons plus myelin sheaths in the horse represent only 37 and 39 per cent of the whole in the medullated median and non-medullated splenic nerves, respectively. It would appear therefore that as much as 50 per cent of the nerve may reasonably be regarded as outside the fibres.

In spite of such theoretical difficulties, however, it seems difficult to avoid these conclusions if one starts with the theory that the fibres are impermeable to anions and to sodium, and permeable only to potassium. The large negativity produced by potassium solutions in measurements of the resting potential in nerve certainly forces one to the conclusion that potassium has a higher mobility in the nerve than chloride. This high mobility is not shared by all cations for NaCl does not show the same effect. The ready diffusibility of chloride and of sodium from the nerve

makes it exceedingly likely that these substances are contained in extracellular spaces. It is difficult further to imagine that the solution in the extracellular spaces differs materially from the blood plasma, although connective tissue cells may of course differ considerably. The data of table 6 are presented, however, not as a certain conclusion but simply as the result which is attained if certain probable assumptions are made.

SUMMARY

1. Frog nerves were soaked for varying periods in different solutions and subsequently analyzed for sodium, potassium, chloride, and bicarbonate.

2. Nerves lose potassium to the solution when the concentration is less than 2 milli-equivalents per cent potassium in March frogs and less than 0.5 milli-equivalent per cent potassium in November frogs. In solutions of higher concentrations of potassium the nerve potassium diffuses into the nerves in spite of the higher concentration inside.

3. The potassium content of nerves is 4.8 milli-equivalents per cent in March and 3.02 milli-equivalents per cent in November.

4. It was not possible to show that potassium diffuses into a nerve more readily from an alkaline solution, as it does in muscle.

5. The pH inside the nerve fibres in vivo is more acid than that outside, but tends to become equal after soaking in Ringer's solution. It becomes more acid after soaking in an acid solution and more alkaline in an alkaline solution.

6. If the chloride content of the solution is varied the chloride content of the nerve varies in proportion, being 50 per cent of that outside in normal nerve and 57 per cent in soaked nerves. Chloride readily exchanges with other anions in the solution and is presumably located outside the nerve fibres.

7. Most of the sodium behaves like chloride but the sodium which is present in excess of the chloride seems to be more firmly fixed in the nerve and is possibly retained inside some of the cells.

8. While soaking in Ringer's solution potassium is lost, partly in exchange for sodium, while chloride is gained probably in exchange with some other anions previously present or formed in metabolism.

9. Chloride and most of the sodium readily escapes from nerves immersed in isotonic sugar solution while the potassium is relatively indiffusible.

10. A tentative distribution of the electrolytes between the inside and outside of the fibres of frog nerve is suggested. According to this scheme approximately 36 per cent of the weight of the nerve is "potassium space" while the remainder is "sodium chloride space." There appears to be a large excess of inorganic cations over anions in the potassium space.

11. In lobster and crab nerves, about 25 per cent of the weight of the

nerve contains sodium and chloride in concentrations equal to those outside. The remainder of the nerve probably contains most of the potassium in concentrations 12 to 15 times as great as that outside.

12. As potassium is lost from the crab and lobster nerves they gain in sodium, calcium, and chloride. The gain in chloride is particularly marked and probably occurs in exchange with some other still undetermined anions originally present.

Since sending this paper to press a paper by S. L. Cowan has appeared (Proc. Roy. Soc. B 115, 216, 1934) in which he gives some analyses of nerves of the crab *Maia*. The total cations he finds equivalent to the total cations in sea water but he states that "preliminary analyses showed that K is the only cation besides Na present in any considerable quantity." This does not agree with our findings according to which the anions are divided fairly equally between K, Na, Ca and probably Mg. Our K values agree fairly well with those of Cowan. Cowan's other results and interpretations seem to be consistent with ours. An earlier paper by Cowan which has just come to our attention (J. Exp. Biol. 10, 401, 1933) indicates that our pH values were not accurately calculated from the CO_2 contents of nerves although the relative values are presumably correct.

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PHOSPHORUS COMPOUNDS IN THE PERFUSED HEART OF THE DOG

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A study of compounds of phosphorus in the cardiac tissue of normal dogs recently has been reported (8). The values are fairly uniform in different dogs even under very different dietary conditions. Although utilization of carbohydrate is accompanied by decrease in the inorganic phosphate of the blood and urine, no evidence has been obtained for a change in the phosphate compounds of the tissue, such as the muscle, which may be using the carbohydrate. In the intact animal the amount of tissue is large; changes may occur and may be too small to detect in any given region. An isolated tissue, the heart in the Starling heart-lung preparation, appeared to be advantageous in a study of the effect of glucose on the phosphorus compounds of cardiac muscle. In a preliminary report (9) based on ten perfusion experiments, attention was called to the fact that when glucose was added to the perfusing blood there was marked increase in the amount of phosphocreatine. More extensive investigation of this problem was carried out to see if glucose was the determining factor.

Normal dogs, in good condition and fasted for eighteen hours, were used. The Starling heart-lung preparation was made with the animal under amytal or ether anesthesia. Differences due to anesthesia were not observed. Defibrinated, heparinized blood was used in all cases. The hearts were perfused for varying intervals ranging from a half hour to five hours, with blood alone or with blood to which one or more of the following substances were added: glucose, insulin and sodium monophosphate or sodium diphosphate. In the early experiments, insufflation was done with air alone or with the addition of oxygen when necessary to maintain arterial color of the perfusing blood. In the later experiments the lungs were aerated, and in addition oxygen was bubbled through the reservoir of blood to insure optimal oxygenation. The procedures involved in analysis of the tissue have been reported previously.

Every precaution was taken to insure optimal conditions for the heart-lung preparation. The largest possible cannulas were placed in the brachiocephalic and left brachial arteries to insure adequate emptying of the

heart. The venous reservoir was kept at a constant level and the temperature of the perfusing blood was maintained at about 37.5°C. Special care was exercised during the preparation and perfusion to prevent overinflation of the lungs, which we have found predisposes them to edema. In spite of these precautions the preparation occasionally showed undesirable manifestations during the period of perfusion. These untoward manifestations were excessive pericardial effusion and extensive ecchymosis.

TABLE 1

Perfused hearts (phosphorus and creatine expressed in milligrams per 100 grams of tissue)

DOG	LENGTH OF PERFUSION, HOURS	TOTAL CREATINE	TOTAL ACID SOLUBLE PHOSPHORUS	Ba SOLUBLE PHOSPHORUS COMPOUNDS	PHOSPHOCREATINE	HEXOSE MONOPHOSPHATE	Ba INSOLUBLE PHOSPHORUS COMPOUNDS	INORGANIC PHOSPHORUS	PYROPHOSPHATE	GLYCOGEN, PER CENT	WATER, PER CENT
Hearts in poor condition with normal phosphocreatine											
1	4	167	61.0	20.0	14.0	3.0	37.0	11.0	11.0	0.40	79.4
2	4	144	68.0	17.0	11.0	2.0	50.0	15.0	19.0	0.30	80.0
3	2	159	74.6	24.1	10.6	9.1	50.0	15.0	15.5	0.58	80.6
4	2*	176	72.9	27.8	10.4	10.6	45.6	12.6	14.6	0.81	79.0
Hearts in good condition with normal phosphocreatine											
1	½	253	124.0	30.0	12.5	12.6	90.6	33.4	38.5	0.62	78.8
2	½	278	122.0	28.2	10.4	13.9	92.0	34.4	35.1	0.66	78.4
3	1½	228	110.2	28.7	11.9	12.2	81.6	24.8	34.7	0.67	80.8
4	2	230	94.8	33.8	13.0	15.3	58.6	20.8	20.6	0.75	79.7
5	2	234	94.2	32.2	13.6	14.2	62.8	17.6	22.8	1.26	79.0
6	2	245	93.2	27.1	9.4	12.7	72.1	28.7	24.6	0.83	81.0
7	2*	211	80.2	21.0	7.2	10.9	60.2	24.4	20.6	0.74	82.3
8	3½	234	96.8	27.2	11.6	12.3	70.8	23.5	28.0	0.80	80.4
9	4	251	119.0	31.3	12.2	11.2	87.4	33.6	32.8	0.61	79.2
10	4*	159	84.2	20.2	11.6	6.9	65.3	17.4	24.7	0.80	76.5
11	4†	233	115.2	21.2	13.1	6.0	90.4	38.4	24.2	0.84	

* Glucose added to perfusing blood.

† Glucose, insulin and phosphate added to perfusing blood.

It is interesting to note that even under these adverse conditions the amount of phosphocreatine (10 to 14 mgm. per 100 gm.) was as high as or slightly higher than that found in hearts which were analyzed immediately on removal from a normal animal. In contrast to this were the low values found for inorganic phosphate and the easily hydrolyzable or pyrophosphate fraction. These low values were reflected in the low values for total acid-soluble phosphate and total phosphate. The amount of total creatine was also low.

The hearts, which remained in good or excellent condition during the perfusion, were arbitrarily divided into two groups: those in which the values for phosphocreatine were about the same as those of normal animals, and those in which the value for phosphocreatine was markedly increased. The fact that high values were not found in the shorter experiments suggests that we were not dealing with the phenomena of resynthesis of phosphocreatine, which existed in the intact animal and was broken down during the setting up of the heart-lung preparation.

In the experiments in which glucose was used, 1 gram was added every half hour to the perfusing blood, which was roughly 1 liter in quantity. When insulin was used, two units were added every half hour, and when phosphate was used 750 mgm. of either sodium phosphate or a mixture of the dibasic and monobasic salt was added at hourly intervals. Blood glucose and phosphate were decidedly decreased at the end of each interval of time after this addition (10). When nothing was added to the blood the value for sugar gradually decreased, usually reaching 20 mgm. per 100 cc. in three hours, with a simultaneous decrease in blood inorganic phosphate. In the experiments in which glucose was added it was often noted that immediately on the addition of the glucose there would be a sudden increase in the strength of the heart beat.

Of the hearts with high phosphocreatine content (16 to 30 mgm. per 100 gm.) the majority were those which were perfused with blood plus glucose. Insulin and phosphate did not seem to have any additional effect. Three of the hearts in this group were perfused with blood only. Most of the hearts in the group in which the partition of compounds of phosphorus more closely resembled those found in the normal animal, were perfused with blood only, although there were a few cases in which glucose was used without an increase in phosphocreatine occurring. In general those hearts that were perfused without additional glucose showed a higher hexose monophosphate than the others.

Comment. Eggelton and Eggelton (1927) showed the presence of small amounts of phosphagen in the cardiac muscle of the frog. The amount was about one-tenth of that found in skeletal tissue. Buell, Strauss and Andrus reported values for the cardiac muscle of cats that were one-sixth of those in skeletal muscle. In the dog we have found (1) normal values for phosphocreatine that are one-fourth as high as those for skeletal tissue. Pohle (1929) proved the presence of adenylic acid and hexose monophosphate in the heart of the ox. Clark, Eggelton and Eggelton studied the compounds of phosphorus in hearts of frogs that were perfused for twenty-four hours with oxygenated Ringer's solution. The changes were surprisingly small. Following its breakdown during contraction the phosphocreatine was readily resynthesized in the presence of oxygen. Later, the investigators (3) showed that ventricles of the frog and of the tortoise

were able to beat for some time in the absence of oxygen, when containing less than 1 mgm. of phosphocreatine per 100 grams. Energy from the anaerobic breakdown of glycogen probably accounted for the restoration of this phosphocreatine following each beat. Meyerhof and Lohmann (1931) in a discussion of the energetics of muscle systems, pointed out that the breakdown of adenosine triphosphoric acid may also supply energy for the resynthesis of phosphocreatine.

In all of our perfusion experiments the phosphocreatine of the heart was as high as, or higher than, that found in hearts removed directly from the intact animal. This is true even of those hearts which obviously are in poor condition at the end of the experiment and in which the inorganic

TABLE 2

Perfused hearts in good condition with high phosphocreatine (phosphorus and creatine expressed in milligrams per 100 grams of tissue)

DOG	LENGTH OF PERFUSION, HOURS	TOTAL CREATINE	TOTAL ACID SOLUBLE PHOSPHORUS	Ba SOLUBLE PHOSPHORUS COMPOUNDS	PHOSPHOCREATINE	HEXOSE MONOPHOSPHATE	Ba INSOLUBLE PHOSPHORUS COMPOUNDS	INORGANIC PHOSPHORUS	PYROPHOSPHATE	GLYCOGEN, PER CENT	WATER, PER CENT
1	1½*	209	101.8	28.6	19.2	7.8	72.2	22.3	26.7	1.22	79.2
2	2¼	338	124.0	31.7	16.8	12.4	96.6	36.9	33.0	0.58	78.8
3	3	245	117.0	45.3	29.8	17.4	71.0	23.4	28.2	1.19	78.8
4	3½*	236	97.8	29.2	19.4	8.4	66.8	17.9	26.6	1.00	78.5
5	4†	215	122.2	30.0	17.4	6.3	82.0	36.0	24.0	0.70	79.8
6	4½†	242	87.7	29.4	18.1	6.0	60.2	20.9	20.6	0.82	79.5
7	5†	211	123.8	38.0	28.0	5.7	90.4	36.9	25.5	1.15	78.4
8	5	246	114.0	35.2	23.6	16.5	81.6	28.4	31.6	0.69	79.3
9	5½†	218	110.6	30.9	22.6	6.5	78.2	27.8	27.4	0.82	77.8
10	‡	181	92.6	28.4	20.4	9.0	64.4	20.4	25.6	0.87	80.7

* Glucose added to perfusing blood.

† Glucose, insulin and phosphate added to perfusing blood.

‡ Glucose and insulin added to perfusing blood.

phosphate, the 10 minute hydrolyzable phosphate or pyrophosphate, and the total creatine, are definitely low.

Eleven hearts which remained in good or excellent condition throughout the experiment showed normal or slightly elevated values for phosphocreatine, with normal or higher values for the other substances analyzed. It is interesting to note that seven of these were perfused for two hours or less. Apparently the building up of unusually high amounts of phosphocreatine is a slow process. It is not merely a restoration of something broken down during the setting up of the heart-lung preparation. In general, these hearts show a striking resemblance to those removed directly from intact animals, although there were a few with higher total acid-soluble phosphorus and higher inorganic phosphorus.

In the group showing high values for phosphocreatine, the length of the perfusion experiment varied from two and a half to five hours. In most of these the phosphocreatine values were from two to three times those found in an intact animal. Seventy per cent of these hearts were perfused with blood to which glucose had been added. If the presence of glucose plays an important part in the production of high values for phosphocreatine, it is difficult to explain the other three cases. In these three, the value for hexosemonophosphates was higher, and it is known that hexosemonophosphates increase as glycogen breaks down (4), and also that the breakdown of glycogen can supply energy for the synthesis of phosphocreatine. The hexosemonophosphate is not elevated in the experiments in which glucose was used. The glucose may be directly or indirectly causing the high values for phosphocreatine in these cases, but because of the presence of other undefined variables in our set-up this possibility cannot be verified as yet. This much is certain: in hearts that are perfused for some time, and appear to be in excellent condition throughout the entire period of perfusion, the values for phosphocreatine may be two to three times those found in normal animals.

SUMMARY

The phosphocreatine of perfused hearts of dogs may remain normal even under adverse conditions when the values for inorganic phosphate, pyrophosphate and total creatine are low. The partition of phosphorus compounds in perfused hearts under good experimental conditions may be very similar to that in intact animals. The amount of phosphocreatine of perfused hearts that are in good condition may be two to three times the amount found in normal animals. This occurs more often in experiments in which the heart is perfused with blood and solution of glucose. However, it may also occur when no glucose is added.

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CHANGES IN THE PHOSPHORUS COMPOUNDS IN THE PERFUSED HIND LIMB OF THE DOG

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It was observed, during the course of perfusion experiments on the isolated hind limb of the dog that there is a decrease in the concentration of serum phosphates (1) when glucose is added, similar to that observed in the intact animal. It was also found that if phosphates were added to the perfusing blood in increments, in addition to glucose, as much as 400 mgm. of phosphorus could be removed from the perfusing blood by the preparation. In an attempt to determine the site of deposition of these phosphates, and the types of compound involved, the perfused muscles were analyzed in conjunction with some of the work on the cardiac musculature (2) by the method previously described (3).

Normal dogs, fasted for eighteen hours, were anesthetized with amytal and the adductor muscles from a fore limb were removed for control analysis. The perfusion of the hind limbs was accomplished in the following manner: 1. A heart-lung preparation was set up after the manner of Starling. 2. The hind limbs were obtained from another animal after cannulation of the aorta and posterior vena cava in the lumbar region. 3. Hemostasis was effected by a mass ligature embracing the lumbar region. The hind limbs were without arterial blood for not longer than one to two minutes. The tissue was perfused with defibrinated heparinized blood. In some cases 3 grams of glucose and 2 units of insulin were added to this at intervals of a half-hour. In other experiments, in addition to the glucose and insulin, 750 mgm. of sodium phosphate, or a mixture of the dibasic and monobasic phosphate, were added at hourly intervals.

Advantages of the hind limb preparation in a study of muscle phosphates are the comparative isolation of muscular tissue and the possibility of taking a specimen before and after the perfusion. Unpublished work from this laboratory shows that the partition of phosphorus compounds in the four legs of the dog is relatively constant so that the tissue from a fore leg can be used as a control for the hind-limb preparation. In a consideration of the results obtained in these experiments it must be remembered that bone and skin tissues are present as well as muscle tissue.

The first striking observation in the experiments in which glucose, insulin and phosphate were used is that in spite of the addition of such large amounts of phosphate to the perfusing blood, sufficient to bring the concentration to 20 mgm. in each 100 cc. at the beginning of every hour,

TABLE 1

Skeletal muscle before and after 4 hours of perfusion

(Phosphorus expressed in milligrams per 100 grams tissue)

	TOTAL ACID SOLUBLE PHOSPHORUS	Ba SOLUBLE PHOSPHORUS COMPOUNDS	PHOSPHOCREATINE	HEXOSE MONOPHOSPHATE	Ba INSOLUBLE PHOSPHORUS COMPOUNDS	INORGANIC PHOSPHORUS	PYROPHOSPHATE	GLYCOGEN, PER CENT	WATER, PER CENT
1†	117	50	38	7	68	29	34	1.90	76
	137	56	44	17	77	32	29	1.64	77
2†	140	43	38	6	94	32	36	0.47	78
	140	54	48	7	83	30	34	1.28	78
3†	114	47	38	6	72	23	24	0.64	78
	142	72	62	5	74	25	26	1.20	75
4†	118	46	38	6	69	27	24	1.84	79
	120	59	42	12	64	20	23	1.11	78
5*	120	50	43	7	70	24	24	0.58	78
	145	54	47	10	84	33	27	1.39	78
6* cyanotic	129	54	41	17	77	26	26	1.66	77.5
	140	39	11	29	96	62	19	1.70	78.8
7	128	48	39	11	73	25	30	0.94	78.8
	146	61	49	13	82	31	32	0.68	78.4
8	127	51	39	9	71	26	29	0.84	76.1
	120	54	45	5	70	26	29	0.49	78.6
9	131	59	41	8	73	28	26		78.9
	132	51	42	8	81	37	26		78.8

* Glucose and insulin added.

† Glucose, insulin and phosphorus added.

there is practically no change in the inorganic phosphate content of the muscle. The loss of phosphate from the blood stream was not a matter of simple diffusion into the tissues. This fact, plus the response of the muscle of the hind leg to an induction current at the end of the period of perfusion, are evidences of the viability of the preparation.

There is a consistent, although in some cases a small, increase in phosphocreatine in these experiments. This seemed to occur regardless of whether the amount of glycogen in the hind limb increased or decreased.

Two hind limb preparations were perfused with blood plus glucose. One of these happened to become cyanotic during the perfusion. The results obtained in this case are similar to those obtained in an intact animal when cyanosis is induced by ligation of the femoral artery. There is a decrease in the phosphocreatine and nucleotide pyrophosphate, and a marked increase in the hexose monophosphate. In the other hind limb preparation, in which glucose only was added to the blood, the results obtained were similar to those found when insulin and phosphate also were used.

Three preparations were perfused with blood only. In one of these there was no increase in phosphocreatine, but the other two showed definite increases. Other factors than glucose must be involved.

SUMMARY

Phosphocreatine may increase during the perfusion of skeletal muscle.

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COMPOUNDS OF PHOSPHORUS IN THE HEART AND STRIATED MUSCLES OF THE DOG: METHODS OF DETERMINATION AND NORMAL VALUES[1]

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The presence of phosphatic compounds in muscular tissue has been known for some time. It is only in more recent years, however, that the relationship between these phosphatic compounds and muscular contraction and carbohydrate utilization has been considered. The contemporary literature on the subject has been excellently reviewed several times (**7, 9–13**). Extensive bibliographies are appended to these articles and nothing would be added by repetition.

On the basis of the presumptive evidence that the inorganic phosphatic compound, which disappears from the blood stream after an injection of a glucose solution is deposited in muscular tissue (14), a series of experiments was planned to demonstrate whether this evidence could be borne out and to determine in what form this phosphatic compound was deposited. In order to keep the experiments comparable, the animal chosen for this work was the dog.

That part of the work reported here involves explanation of the analytical methods used and a report of the normal values for selected phosphatic compounds in the striated and cardiac musculature of the dog.

METHODS. Male and female mongrel dogs were used exclusively. The animals were in good health at the time of the experiment. Amytal was the anesthetic agent employed throughout this series. The animals were anesthetized, the skin was shaved and an incision made. The muscle selected for the experiment was then freed along the lines of cleavage, leaving the blood and nerve supply intact. At the proper time the muscle was rapidly severed from its remaining connections and dropped into a freezing mixture of carbon dioxide snow and alcohol. It might be mentioned at this point that mixtures of carbon dioxide snow and ether are unsatisfactory because of penetration of ether into the tissue with resulting inconstant weights.

[1] Extract of thesis submitted by Doctor Pollack to the Faculty of the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

The technic for removal of the hearts was modified during the course of the experiments. The first hearts analyzed were removed as rapidly as possible after sectioning the pleura. This technic gave variable results, as is indicated later. The explanation for this was found in the apneic period following pleural section, and its resultant partial anoxemia. When artificial respiration through a tracheal tube was introduced by means of a mechanical pump, previous to the time the pleural cavity was opened, this period of partial anoxemia was avoided. The analyses of the hearts removed after introduction of this modification yielded more consistent and somewhat higher values.

The analytical procedures were then carried out for all tissues alike. The completely frozen tissue was shattered into coarse lumps by means of a mallet. The grossly tendinous portions were removed and discarded. In the case of the hearts, musculature from the left ventricle was used. This and subsequent procedures were carried out in a cold room, the temperature of which was kept close to 0°C. The cleaned tissue was then ground through a fine meat grinder that previously had been chilled. The ground muscle was never allowed to thaw, but was collected in clean porcelain vessels immersed in a bath of alcohol and carbon dioxide snow. Aliquot parts, of about 10 grams each, were then weighed on a semi-analytical balance. Duplicate extractions and duplicate subsequent analyses were run as a routine on separately tared specimens.

The weighed tissue was transferred through a large-mouthed funnel the stem of which was blown to fit the neck of a centrifuge bottle of a capacity of 250 cc. In this container, the temperature of which was slightly below 0°C. was a previously buretted quantity of 5 per cent trichloracetic acid and a few ounces of solid glass beads. After transferring the tissue, the rest of the calculated amount of 5 per cent trichloracetic acid was run in from a burette. The amount of acid used was sufficient to make a 1:10 dilution, estimating water in tissue as 75 per cent. In the final calculations the weight of the tissue and the volume of acid were both taken into account in the equations.

The bottles were tightly stoppered and then fastened to the shaking machine. The extraction time was determined by preliminary experiments and found to be optimal at about fifteen minutes. Checks on the temperature of solutions and extracts at various times always showed a margin of safety below 0°C. After the tissue was thoroughly macerated and extracted on the shaking-machine, the mixture was filtered by suction.

The filtrates obtained from striated muscle were clear as water. Those obtained from cardiac muscle contained very slight traces of a brownish pigment which did not seem to interfere with subsequent procedures. Aliquot parts, 40 cc., of the filtrate were pipetted into chilled centrifuge tubes of a capacity of 50 cc. and immediately neutralized with powdered

barium oxide, using phenolphthalein as an indicator (Eggeltons). This neutralization served two purposes: 1, to alkalinize the solution and retard hydrolysis of the phosphocreatine, and 2, to precipitate the free inorganic orthophosphate ions and the nucleotide pyrophosphate. The phosphocreatine and hexose phosphate remained in solution. The mixture was centrifugalized. The supernatant fluid was decanted into a volumetric flask of a capacity of 50 cc. To recover any part of the barium soluble fraction which may have been carried down by this precipitation, the residue was dissolved in 10 per cent trichloracetic acid and reprecipitated with barium (1). The mixture was centrifugalized again and the supernatant fluid added to that obtained on the first centrifugation. The filtrate will be designated "fraction A" and the precipitate, "fraction B." All final determinations of phosphate in the orthoinorganic form were made by the method of Fiske and Subbarow.

A wet method of ashing was used in determining the total phosphorus of the original extract and the fractions. The one used for this purpose was the superoxol-sulphuric acid method. In a pyrex ashing tube a calculated amount of sulphuric acid was added to an aliquot part of the solution to be ashed. The amount to be added was determined on the basis of the amount of sulphuric acid required for the Fiske and Subbarrow phosphate determination. The mixture was evaporated down beyond the point of charring until white fumes appeared. A half cubic centimeter of superoxol was run in slowly after cooling had been allowed. The mixture was then boiled again until fumes appeared. Usually, subsequent charring did not occur. If the molybdate method is to be used for phosphate determinations on this solution, care must be taken to get rid of excess peroxide. After a clear solution is obtained, water is added and the mixture boiled for ten minutes to convert the phosphate present to the orthophosphatic form.

For ashing of the tissue directly, advantage was taken of the method recently described by Power. Essentially, this consists of ashing in distilled, fuming nitric acid in a fused quartz vessel. The system is closed by a modified Hopkins condenser, so arranged that the distilled acid acts as a trap. Glycogen was determined by the Pflüger method with modifications. Water was determined by drying the tissue to constant weight in an oven at 80°C. Creatine was determined by the method of Folin.

Fraction A was immediately analyzed for labile phosphorus. An aliquot portion of the filtrate was allowed to stand thirty minutes at room temperature after the addition of the calculated amount of the acid molybdate solution. At the end of this period of time the naptholsulphonic acid reagent was added and the mixture made up to volume. A standard was prepared simultaneously with the unknown. The color was allowed to develop for fifteen minutes, and the standard and unknown were compared in a colorimeter.

In Cori's method for hexose phosphate, an aliquot portion of the original trichloracetic acid extract is allowed to stand at room temperature overnight (15°C. for 12 hours). This is done to allow for hydrolysis of the phosphocreatine. They recognized, however, that some breaking down of the adenosine triphosphoric acid also occurs. In the next step of their procedure, barium precipitation is carried out and the supernatant liquid then contains only the hexose phosphate plus a small amount of the ribose phosphate from the nucleotide. Then an alcoholic precipitation was carried out, the precipitate containing the hexose and ribose phosphate. In the experiments reported in this paper the procedure was modified. An aliquot portion of fraction A, which had been purified of its nucleotide phosphate by barium precipitation, was mixed with 8 volumes of ammoniacal alcohol in the presence of the barium and allowed to stand in a refrigerator several hours. The precipitate containing both phosphocreatine and the Embden ester was obtained by centrifugalization. The precipitate was then dissolved in dilute hydrochloric acid. The barium was removed by the addition of just an adequate amount of sulphuric acid. The barium sulphate was removed by centrifugalization and the solution made up to volume. An aliquot portion of this solution was ashed to determine the total phosphorus content. Another aliquot portion was used for the estimation of the labile phosphate (phosphocreatine). Creatine determinations were then carried out on a third aliquot portion. This is a decided advantage as it enables the investigator to calculate the ratio of creatine to phosphorus as a means of checking the type of compound present. Total determinations of creatine were also made both of the original specimens of muscle and the extract of trichloracetic acid. The values for hexose monophosphate represent the difference between the labile phosphate and the total phosphate determinations.

Fraction B was dissolved in dilute hydrochloric acid and made up to a volume of 50 cc. The free phosphate ions were immediately determined. This represents the inorganic phosphate of the extract of muscle. An aliquot portion of B was boiled with normal sulphuric acid for ten minutes according to Lohman. This procedure served to split off the pyrophosphate and convert it into the orthophosphate, which was then determined. An aliquot portion of B was then ashed to determine the total amount of phosphorus in this fraction.

For the purpose of checking the ashing and fractionations, the original trichloracetic acid extract and its fractions were subjected to hydrolysis. The technic employed here was to mix equal volumes of twice normal sulphuric acid and the solution in the mixture. The tubes were closed with glass bulbs, and placed in a beaker of boiling water for the desired length of time. It should be mentioned here that the use of tin foil for closing the tubes must be avoided. The traces of this metal which are

washed back into the solution by the hot condensing steam are sufficient to upset the Fiske and Subbarrow phosphate determinations. In fact, methods for the estimation of phosphate ions have been described which utilize tin salts (3).

EXPERIMENTAL DATA. The results of the analyses of adductor muscles from the hind legs of twelve normal dogs after a fast of eighteen hours are shown in table 1. The total acid soluble phosphorus varies from 114 mgm. to 142 mgm. per 100 grams of muscular tissue. The total phosphorus, including phospholipins and protein phosphorus, shows less variation and ranges from 185 to 213 mgm. per 100 grams. It is to be noted, however, that in spite of the variation in total acid soluble phosphorus, the values of

TABLE 1

Normal muscles

Phosphorus expressed in milligrams per 100 grams of tissue

TOTAL PHOSPHORUS	TOTAL ACID SOLUBLE PHOSPHORUS	TOTAL FRACTION A	PHOSPHOCREATINE	HEXOSE-MONOPHOSPHATE	TOTAL FRACTION B	INORGANIC PHOSPHORUS	10 MINUTE HYDROLYSIS	GLYCOGEN, PER CENT	WATER, PER CENT
206	142	57	44	9	94	30	29	0.82	76.0
192	140	45	32	9	95	39	30		72.0
200	137	46	33	9	89	38	32		76.0
185	135	53	38	7	87	29	34	1.34	72.0
	118	42	39	4	77	32	36	0.87	
213	118	45	39	7	68	29	34	1.92	
200	140	43	38	5	94	34	34	0.47	77.5
	130	44	35	5	80	32	34		
197	114	47	38	6	71	23	26	0.64	77.8
	118	46	38	6	69	27	24	1.85	79.0
	120	50	43	7	70	24	25	0.57	78.0
	129	54	41	7	77	26	26	1.65	76.0

phosphocreatine phosphorus are quite constant, with variations of from 32 to 44 mgm. but in nine of twelve determinations it ranged between 38 and 44 mgm. The sum of fraction A plus fraction B values, which are determined by separate ashing processes, agrees very closely with the value for the total acid soluble phosphorus also obtained by independent ashing. It may be seen, then, that by the methods of fractionation and ashing used the values obtained were well within a 10 per cent difference.

Fraction A was analyzed for phosphocreatine and the Embden ester. The sum of these two compounds accounts for the total fraction in many cases (table 2). However, if one considers experimental error, these results do not rule out traces of an alcohol soluble compound, as has been suggested by other writers. The fact that the values obtained from direct

ashings are generally higher than those obtained by adding the values of the known constituents, does not add any weight to the hypothesis of the presence of an alcohol-soluble compound. It is obvious that the loss will

TABLE 2

Partition of phosphorus compounds not precipitated by barium hydroxide

Phosphorus expressed in milligrams per 100 grams of tissue

PHOSPHOCREATINE	HEXOSEMONOPHOSPHATE	SUM OF PHOSPHOCREATINE AND HEXOSEMONOPHOSPHATE	OBSERVED VALUE	DIFFERENCE
44	9	53	57	4
32	9	41	45	4
33	9	42	46	4
38	7	45	53	8
31	4	43	42	−1
39	7	46	45	−1
38	5	43	43	0
35	5	40	44	4
38	6	44	47	3
38	6	44	46	2
43	7	50	50	0
41	7	48	54	6

TABLE 3

Partition of phosphorus compounds precipitated by barium hydroxide

Phosphorus expressed in milligrams per 100 grams of tissue

TOTAL FRACTION B	INORGANIC PHOSPHORUS	TOTAL NUCLEOTIDE PHOSPHORUS	AMOUNT OF PYROPHOSPHORUS	TOTAL NUCLEOTIDE PHOSPHORUS MINUS PYROPHOSPHORUS
94	30	64	29	35
95	39	56	30	26
89	38	51	32	19
87	29	58	34	24
77	32	45	36	9
68	29	39	34	5
94	34	60	34	26
80	32	48	34	14
71	23	48	21	27
69	27	42	24	18
70	24	46	25	21
71	26	51	26	25

occur in the manipulations and, hence, in the analysis of the hexose monophosphate fraction, for which precipitation from large volumes is required. It may be noted that the values for this hexose monophosphate compound are somewhat higher in the first few than in the later experiments; the

difference, however, is very small and may be accounted for by improvement in technic.

Fraction B presents the most variable values, for reasons that are at present unknown. The amount of orthoinorganic phosphorus is not the cause of this variation, as might be suspected. The variations occur in that portion of the fraction that is said to be the nucleotide pyrophosphate and, more specifically, in the remaining phosphate of the nucleotide after the pyrophosphate has been removed by hydrolysis. This is shown in table 3 in which it is readily seen that the amount of pyrophosphate is fairly uniform. The last column in the table represents the difference beteen the total amount of nucleotide phosphorus (obtained by subtracting the orthoinorganic from the total fraction B phosphorus) and the phosphorus that was present in the pyro form. Theoretically, this quantity should be the phosphorus present in the ribose phosphate molecule. The ideal quantity of this portion of the fraction is calculated on the basis of the nucleotide molecule containing three atoms of phosphorus, one of which is the phosphorus in the ribose phosphate, and the other two, those originally present as the pyro form. Therefore, the rest phosphorus of the fraction B should be equal to one-half of the pyrophosphorus, a conclusion that is not borne out by our results as shown in table 4. The meaning of this failure of our results to agree with those theoretically anticipated in the literature is not evident at present. The whole calculation of the rest phosphorus is dependent on the ashing of the total fraction B. It might be considered a result of experimental errors in the procedure,

TABLE 4

Comparison of undetermined phosphorus as determined with amount calculated on assumption that all organic phosphorus in fraction B is adenyl pyrophosphate

Phosphorus expressed in milligrams per 100 grams of tissue

AMOUNT OF PYROPHOSPHORUS	CALCULATED RIBOSE PHOSPHORUS	OBSERVED REST PHOSPHORUS	DIFFERENCE BETWEEN OBSERVED AND CALCULATED
29	14.5	35	21.5
30	15	26	11
32	16	19	3
34	17	24	7
36	18	9	−9
34	17	5	−12
34	17	26	9
34	17	14	−3
21	10.5	27	16.5
24	12	18	6
25	12.5	21	8.5
26	13	25	12

were it not for the perfect agreement of the values for this total fraction determination with the other fraction and the total acid-soluble extract. There is one justification for such variations: the theoretical calculations for the amount of ribose phosphate are based on the assumption that the nucleotide present is only the adenyl pyrophosphate whose structure calls for two atoms of phosphorus in the pyro form and one atom of phosphorus in the ribose form. This assumption is open to criticism. Adenylic acid is only one of the four nucleotides that comprises the tetranucleotide of nucleic acid. The others are similar compounds containing cytosine, thymine and guanine, instead of adenine. It is conceivable that these might also exist as mononucleotides and thus be found in this combined with pyrophosphates, fraction of the acid extract. In fact, this fraction

TABLE 5

Normal hearts

Phosphorus expressed in milligrams per 100 grams of tissue

TOTAL PHOSPHORUS	TOTAL ACID SOLUBLE PHOSPHORUS	TOTAL FRACTION A	PHOSPHOCREATINE	HEXOSE-MONOPHOSPHATE	TOTAL FRACTION B	INORGANIC PHOSPHORUS	10 MINUTE HYDROLYSIS	GLYCOGEN, PER CENT	WATER, PER CENT
221	90	16	Trace	10	68	26	26	0.50	77.0
216	103	11	Trace	8	91	34	28	1.00	
	90	14	6.5	8	72	23	28		
	81	13	3	8	64	24	20	1.05	79.0
	83	19	7	8	65	21	25	0.80	77.8
	100	21	8.5	10	79	28	30	0.74	78.0
*	95	20	11	8	76	29	31	0.95	77.0
209*	108	21	8	10	82	28	35	0.92	77.0
205*	110	22	8	11	84	33	33	0.76	78.0

* Artificial respiration.

gives a positive reaction in Wheeler and Johnson's color test for cytosine and uracil. Further work on identification of the variable part of fraction B is in progress.

The values obtained from the analyses of the hearts are shown in table 5. The first six analyses were of hearts removed from the dogs under amytal anesthesia, without attention to the respiratory cycle. It will be noted that the values for the phosphocreatine are very variable, ranging from traces up to 8.5 mgm. per 100 grams of tissue. This variation is in agreement with the values for this compound as reported in the literature. It seemed to us, however, that an error in technic must be responsible for such a wide variation. An analysis of the technic used for removing the tissues brought out a salient point. At the time the pleura is sectioned, there is a period of apnea that produces partial anoxemia. The technic

TABLE 6

Normal muscles. Hydrolysis of total acid-soluble phosphorus (TAS) and fractions A and B in 1 normal sulphuric acid at 100°C.

Phosphorus expressed in milligrams per 100 grams of tissue

	0	10 MINUTES	40 MINUTES	1 HOUR	2 HOURS	3 HOURS	4 HOURS	TOTAL
TAS	73	120	128	133		132	128	142
A	44							57
B	30	59						94
TAS		101	117	115		125	134	140
A	32	33	32	33				45
B	39	77	84		84			95
TAS		97	110	111		122	129	137
A	33	33	33	32				46
B	38	78	76	79				89
TAS		115	112	116	113	124	133	135
A	38							53
B	29	63						87
TAS		101	108	111	114	114	119	118
A	39							42
B	32	68						77
TAS	77	97	102	103	104	106	111	118
A	39	40		43				45
B	29	59		60				68
TAS	72			116			125	140
A	38	39		41				43
B	34	68		73				94
TAS	71						124	130
A	35	38		40			40	44
B	32	64		72			75	80
TAS	64	94	99	96	99		109	114
A	38	40		42			42	47
B	23	49		54			59	71
TAS	69			102			106	118
A	38	40	43	43				46
B	27	51		57			62	69
TAS	72						112	120
A	43	48	48	47				50
B	24	49		54			59	70
TAS	68				107		112	129
A	41	47		49				54
B	26	52		58			63	77

was improved by instituting artificial respiration before any operative manipulations were started. This was done by means of a tracheal cannula and a mechanical pump. The last three analyses shown in table 6 were of hearts removed under artificial respiration. The phosphocreatine values are higher and more consistent.

The differences between the values for the total acid-soluble phosphorus, as obtained from a direct ashing of the original trichloracetic acid extract,

TABLE 7

Normal hearts. Hydrolysis of total acid-soluble phosphorus (TAS) and fractions A and B in 1 normal sulphuric acid at 100°C.

Phosphorus expressed in milligrams per 100 grams of tissue

	0	10 MINUTES	40 MINUTES	1 HOUR	2 HOURS	3 HOURS	4 HOURS	TOTAL
TAS	38	61	66	68	69	71	79	90
A	Trace	Trace		5.6				16
B	26	53		56				68
TAS	35.5			74.1			82	103
A	Trace	Trace		Trace				11
B	34	62		64				91
TAS	31						76	90
A	6.5	8		8.5			9.7	14
B	23	49		55			62	72
TAS	30			59			61	81
A	3	8	5	6				13
B	24	44		52			55	65
TAS	30			59			70	83
A	7	8	10	10				19
B	21	46		52			55	65
TAS	36				77		82	100
A	8.5	10		12				21
B	28	58		64			69	79

and that obtained by adding the separate fractionation values are small. The total phosphocreatine of the heart is less than that observed in the striated muscle. The hexose phosphate fraction, however, averages slightly higher in cardiac muscle. The values obtained from the ashings of the fraction as a whole agree with those obtained from adding the constituent parts. The variations are almost as great for constituents precipitated by barium hydroxide from the cardiac tissue as for the skeletal muscle. It can be seen that the pyrophosphate fraction as well as the

orthoinorganic phosphate fraction are fairly uniform in both muscle and heart. There is slightly greater consistency in the calculated amount of undetermined phosphorus in the heart. It will be noted that in experiments on the hearts removed under artificial respiration the differences between the observed undetermined phosphorus and that calculated for ribose phosphate are within limits of experimental error, although other substances containing phosphorus may be present in small amounts, for we find that a positive reaction in Wheeler and Johnson's color test for cytosine and uracil similar to that found in skeletal muscle is obtained with this fraction of cardiac tissue. Recently, Drury has reported the presence of a nucleotide in the cow's heart that differs from adenine nucleotide in its physiologic effect on hearts of rats and in its slower hydrolysis in mineral acids. He suggested the possibility that this was cytidylic acid.

Data obtained from the hydrolysis experiments are given in tables 6 and 7. All experiments of this type were consistent with each other and with the results of ashing and fractionation. The interest in these figures lies mainly in their confirmatory evidence. In this way, also, a check is obtained on the type of compound present; any deviation of the hydrolysis curve from that of the control types would lead one to suspect the presence of a different compound. The major part of the increase in orthoinorganic phosphate during hydrolysis is during the first ten minutes. The major part of the increase is in fraction B and presumably is due to conversion of the pyrophosphate to the orthoinorganic phosphate. A small increase takes place in fraction A also. From the ten to forty minute period there is an increment in fraction B that usually appears to be about 6 mgm. From the first to the third hour no change of significance can be noted. During the fourth hour of hydrolysis there is always some change. This fractional increase may again be located in fraction B. In fraction A there are a few milligrams of a phosphate compound that does not hydrolyze in four hours. In fraction B there is a greater amount of organic phosphorus that does not hydrolyze in four hours.

SUMMARY

Values are presented for the acid-soluble phosphate compounds of cardiac and skeletal tissue in the normal dog.

Fraction A, which is not precipitated by barium salts, is composed chiefly of phosphocreatine and hexose monophosphate, but there may be also a small amount of a compound that is soluble in the presence of alcohol and barium hydroxide.

In cardiac tissue, the phosphocreatine may occur in amounts as high as one-third of that in skeletal tissue.

Fraction B, which is precipitated by barium salts, contains inorganic phosphorus and organic phosphorus. A large part of the latter can be

accounted for as adenylpyrophosphate. There is a significant amount left to be accounted for, probably consisting of other mononucleotides. In both cardiac and skeletal tissues of the dog, this fraction produces a positive reaction in Wheeler and Johnson's color test for cytosine and uracil.

Values for total phosphates are about the same in amount in both skeletal and cardiac tissue. A larger proportion of the total phosphates in the skeletal tissue is acid-soluble.

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SERUM PHOSPHATE CHANGES INDUCED BY INJECTIONS OF GLUCOSE INTO DOGS UNDER VARIOUS CONDITIONS

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When glucose is given intravenously or by mouth, in sufficient quantities to produce hyperglycemia in normal people or animals, there is a decrease in the concentration of inorganic phosphate in the serum. Fiske (3) was the first to call attention to this specific relationship between carbohydrates and phosphates. He showed that the post-prandial retention of phosphate was not due to the alkaline tide, but was related to carbohydrates in the diet. Perlzweig, Latham, and Keefer (6) demonstrated the changes in serum phosphate after injection of insulin as well as of glucose. These observations were amply confirmed by Harrop and Benedict (4), as well as by Bolliger and Hartman (2). Sokhey and Allan (7) carried out extensive research on this problem and amplified as well as extended the then existing facts. They found a depression of excretion of urinary phosphates after injection of glucose almost as great as after injection of insulin. Allan and others (1) further demonstrated that epinephrine was not antagonistic to insulin in regard to the depression of concentrations of serum phosphate as it was in effect on levels of blood sugar. Markowitz (5), from the same laboratory, rounded out the series of experiments by proving the necessity of insulin in this reaction.

It seemed that a study of the site of deposition of the phosphate, as well as the type of compound formed, might give an insight into the intermediary carbohydrate metabolism. To this end the following series of experiments was planned and carried out. The technic, in brief, is as follows: Female, mongrel dogs, about 15 kgm. in weight, were trained to lie quietly. Solutions of glucose were injected continuously, intravenously, at the rate of 2 grams for each kilogram of body weight throughout every hour. The injection was controlled by a Woodyatt pump. The driving force of the pump was a synchronous, single phase motor of a capacity of one-tenth horse power. This type of driving force insures greater accuracy in the rate of injection. Determinations of glucose and phosphate in the blood and urine were carried out, as well as some observations on the lactic acid changes.

The unlaked filtrate of Folin was used for determinations of blood sugar.

The method of Fiske and Subbarow was used for estimations of phosphate. Lactic acid was determined by the Friedman, Cotonio, Shaffer technic. Concentrations of sugar in urine were measured by the Shaffer-Hartman method after suitable dilution.

All operations were performed under ether anesthesia and with aseptic technic.

The results of the experiments can be summarized very briefly. In the normal animal, within two hours after the beginning of injection of

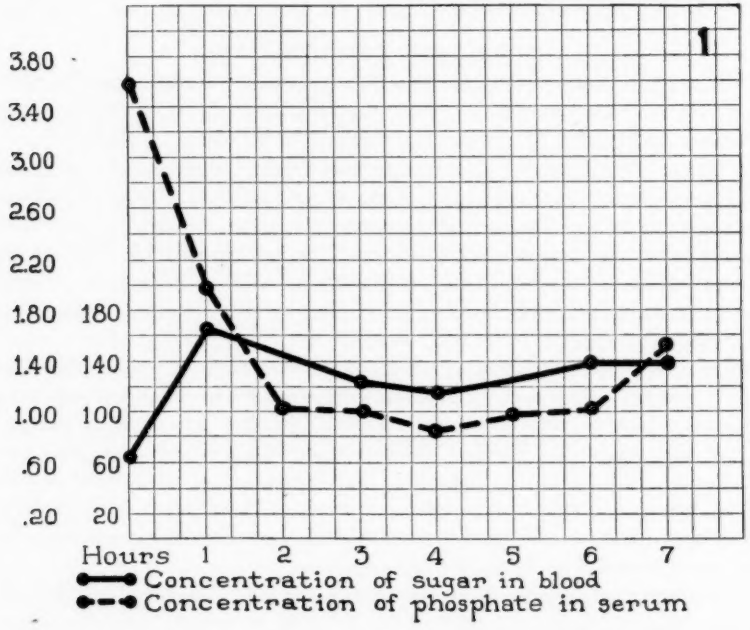

Fig. 1. Concentrations of blood sugar and serum phosphate in hourly periods. Normal dog receiving 2 grams of glucose for each kilogram of body weight each hour.

glucose, the serum phosphates will have dropped from a normal level of between 3 and 4 mgm. in each 100 cc. down to from traces to 1 mgm. in each 100 cc. This low level is maintained for from fourteen to eighteen hours, at which time, in spite of the continued intravenous injection of glucose, there is a slow rise to the normal resting values. The concentrations of urinary phosphate parallel those of the serum (fig. 1).

In a series of hepatectomized animals a similar phenomenon was observed. The only differences between the curves of these two series were that the decrease in concentration of serum phosphate occurred more rapidly, and returned to normal levels sooner (fig. 2).

Adrenalectomized animals were then observed with the same technic. These animals did not survive the experiment long enough to show a return to normal levels. That part of the curve which was obtained, however, did not differ from the normal.

Dogs, made diabetic by pancreatectomy, were given injections of glucose. No change in serum phosphates occurred unless insulin was supplied with the injection.

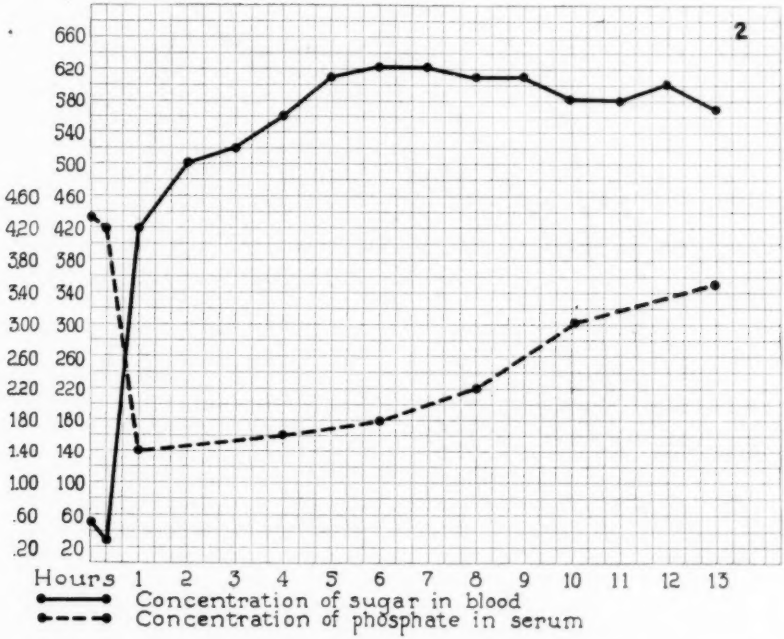

Fig. 2. Concentrations of blood sugar and serum phosphate in hourly periods. Hepatectomized dog receiving 2 grams glucose for each kilogram of body weight each hour.

In order to localize further the site of deposition of this phosphate, recourse was had to a preparation from which all the striated muscle had been removed. For this type of experiment the visceral organism of Markowitz and Essex was chosen. Glucose was given by means of continuous injection, directly into the superior vena cava. In a good preparation, maintaining a constant blood sugar level there is no decrease in concentration of serum phosphate (fig. 3).

As a final step in this series of experiments, isolated hind limbs of a dog

were perfused. The Starling heart-lung preparation was used as the pump for the circulating blood. With this preparation, a marked decrease was noted in the concentration of phosphate in the circulating blood. Figure 4

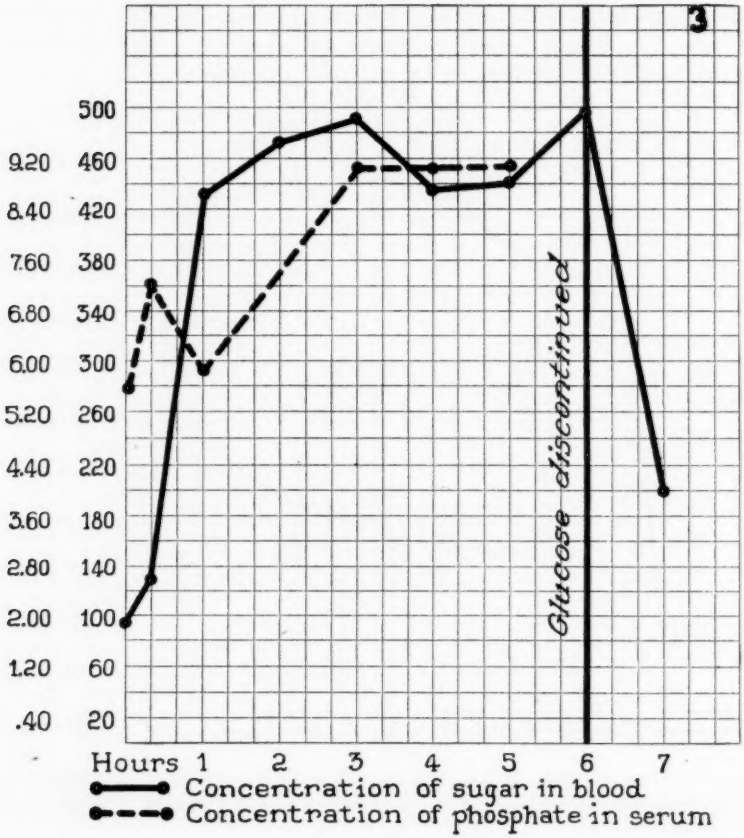

Fig. 3. Concentrations of blood sugar and serum phosphate. Visceral organism preparation, receiving 1 gram of glucose in each hour by continuous injection.

illustrates a typical four-hour experiment with this preparation. At the end of each hour, sodium phosphate (mixture of monobasic and dibasic) was added in fixed increments as well as glucose. It is to be noted that the successive hours show essentially the same type of curve.

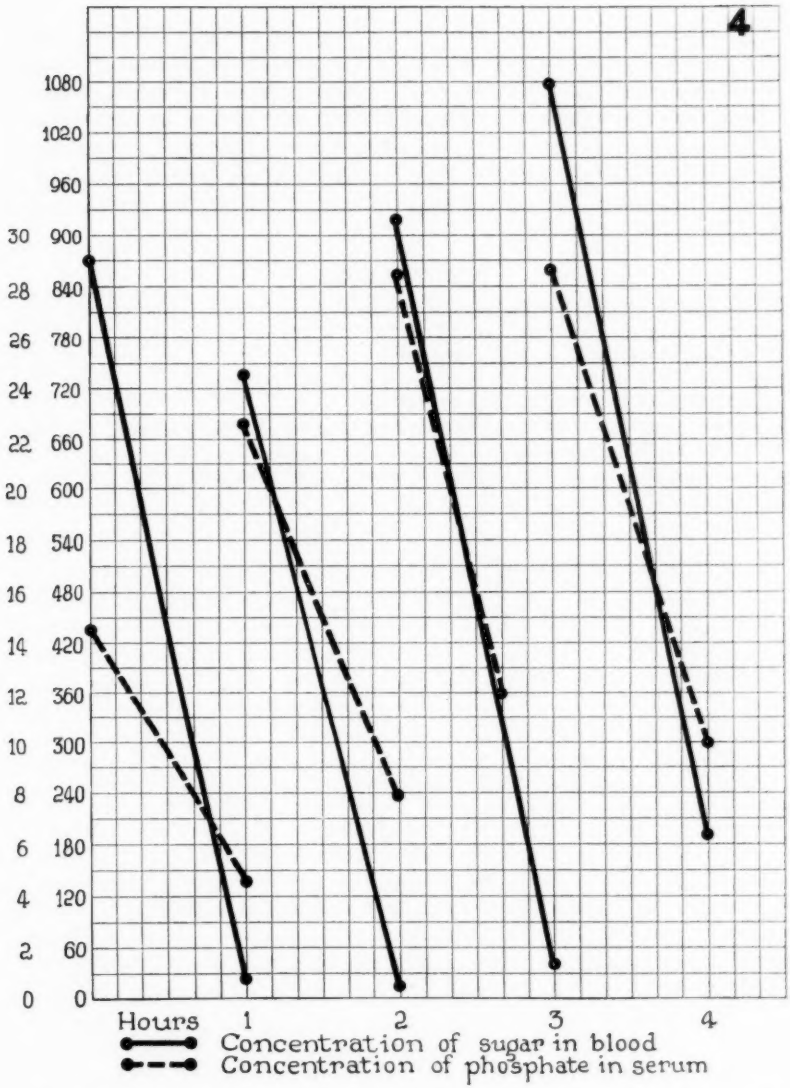

Fig. 4. Blood sugar and serum phosphate determined on the perfusing blood of a heart-lung-hind-limb preparation. The high values at the beginning of each hour represent the increase in concentration after the addition of known amounts of sodium phosphate and glucose.

CONCLUSIONS

It has been shown that the serum phosphate changes following intravenous injections of glucose seem to have a capacity time factor. That is, after a definite length of time of the injection period, there is a return to the normal concentrations. The liver does not seem to be concerned in this reaction, as is shown by the presence of the reaction in the absence of the liver and the absence of the change when the liver is present in a visceral organism. The site of deposition of this phosphate is presumably in the muscle.

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INTESTINAL ACTIVITY IN THE EXTERIORIZED COLON OF THE DOG

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The experiments described herewith involve primarily the exteriorization of the intestine, so that *a*, the movements of the circular and longitudinal muscle may be observed and recorded; and *b*, so that the presenting mucosal surface may be acted upon artificially in a manner approaching intra-intestinal conditions. The bowel segment retains its original blood and nerve supply until such time as these are severed by a second operation.

Method of preparing the animal. Medium sized female dogs were used. Under ether anesthesia and surgical aseptic technic, the bowel segment (herein the distal transverse colon) is pulled out of the abdominal cavity. A loop of about 8 to 10 cm. is cut away from the colon. Continuity is reestablished by an end to end anastamosis, and the colon is returned to the abdominal cavity. The resected loop is then pulled out of the abdomen, and the peritoneum and muscles are sewn around the mesenteric pedicle, sufficient space (2 cm.) being left to prevent strangulation. The isolated intestinal loop is cut longitudinally, along the anti-mesenteric border. Fecal material is washed away, and the mucosa kept moist with saline. An anchor suture is placed in each corner of the intestinal flap, to hold it open. The approximate area which the intestinal flap is to occupy is now measured off on the surface of the abdomen, and a corresponding amount of skin is removed, with as little subcutaneous tissue as possible. Finally the flap is fixed into place by suturing together the serosa and subcutaneous tissues at the mesenteric border, and also at the periphery, on all four sides, with the proximal border of the graft being placed cephalad. The suture includes the mucosa and serosa, and the skin and subcutaneous tissues. Vaseline packs held in place with an apron minimize trauma. The animal is ready for use within 8 to 10 days after operation. The healthy transplant is shown in the accompanying photograph, figure 1.

The animals are now trained to lie quietly, without undue movement. Quiet is essential, as the least movement of the animal results in displacement of the recording lever, and may mar the tracings.

The recording apparatus. This is a modified enterograph lever system

of two separate units, one for the longitudinal (I, fig. 2) and another for the circular (II, fig. 2) muscle.[1] The spring clips *A* and *B* attach onto the mucosa of the region of the graft the movements of which are to be recorded. The steel rods *F* and *G* serve as leverage, through the fulcrum brass bar *J*. One steel rod, which is free to move on a hinge (*C*) at its attachment with

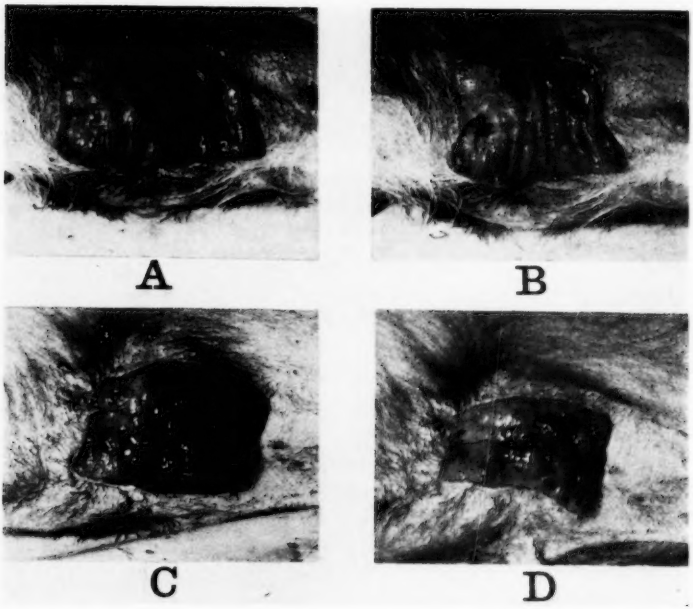

Fig. 1. Unretouched photograph of the colonic graft in one of the dogs. The left of the picture is proximal.

A. Appearance at rest.

B. The mucosa was stroked proximo-distally, with a metal teaser. Notice the shortening and widening of the graft, which indicates contraction of the longitudinal muscle.

C. The mucosa was stroked proximally in a transverse direction. There is contraction of the circular muscle only at the site of stimulus application.

D. The mucosa is stroked transversely along its whole length by a series of strokes. Note the graft has become narrower and longer. Contrast with B above.

the bar *J*, serves to motivate the writing lever (not shown) by means of the string *O*. The remaining rod is not movable, but serves as the fixed point (*D*), of the lever system. Bar *J* hangs from a universal joint *M* by a rod *L*, along which it may be adjusted to any desirable height, to fit

[1] Made by Joseph Becker, New York.

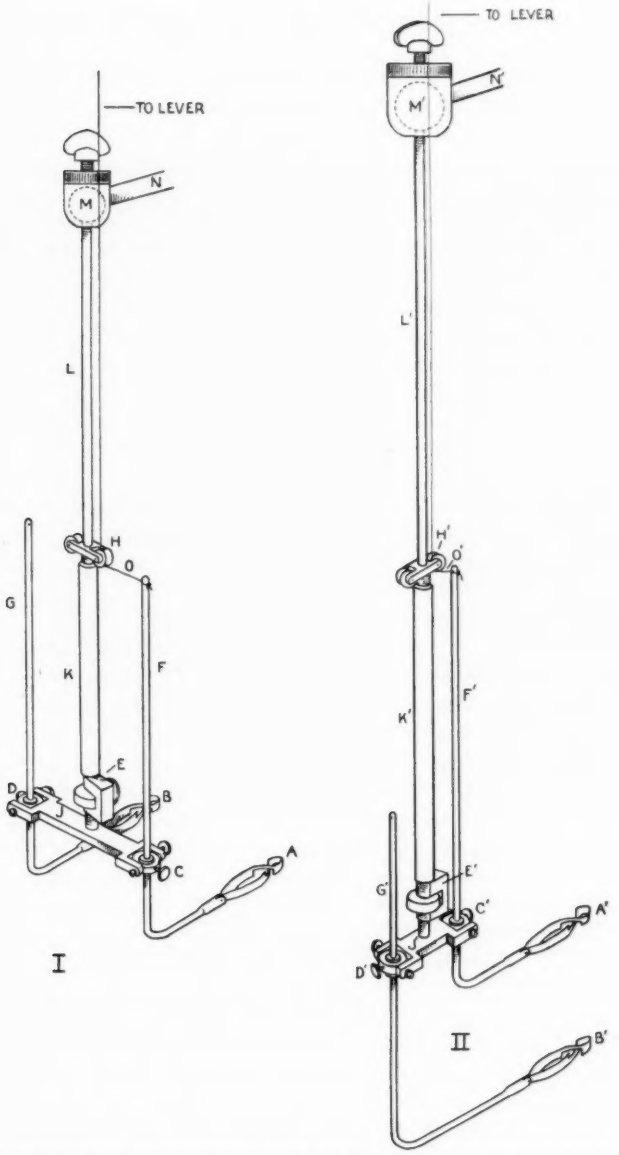

Fig. 2. Drawing of the apparatus used for making the records in figure 3.

I. The element used for recording the contractions of the longitudinal muscle.

II. The element used for recording the circular movements. For full description, see text.

individual animals. The whole apparatus is fixed by rod *N* to a rigid stand.

Any contraction of the graft will shorten the distance between *A* and *B*, and this movement will be transmitted to the lever by means of the string *O*. Suitable magnification is obtained first by altering the distances between *C* or *D*, and the elbow of the rods *F* or *G*, and secondly by altering the writing lever attachment. The unit II is a replica of the first, except for the vertical arrangement of the clips. It is superimposed upon unit I, without interfering with its action. The height of the movements of the writing levers is a function of the position of the clips on the intestinal graft. This distance cannot be gauged accurately, from day to day, so that the height of the contractions cannot be compared, except from time to time, during any one experiment.

The stimulus. The results of our experiments are based upon the simple mechanical stimulus of stroking the mucosa of the graft with a blunt metal teaser. This is not so sharp as to injure the mucosa.

RESULTS. The application of the spring clips to the mucosa initiates a series of contractions, especially of the longitudinal muscle. These contractions last for from 3 to 10 minutes, and disappear without further influencing the evenness of the tracing. The transverse attachment (circular muscle) oscillates slightly with each respiration, but this offers no real problem in the interpretation of the results.

a. *Resting activity.* If the animal had not been fed within 12 hours or longer, the series of strong contractions which are initiated by the application of the clips to the mucosa soon die down and disappear. Within several hours after the animal had been fed, however, the initial clip contractions may be followed by rhythmic contractions, more marked in the circular muscle. These come on at intervals between 30 seconds to 1 minute, and may last throughout the period of the experiment (2 hours).

b. *Stroke stimulus applied longitudinally* (fig. 3). The longitudinally recording clips were attached the whole length of the strip, and the stimulus was applied between the clips. The transverse clips were applied to the center of the longitudinal borders. In all of these experiments the stimulus is of from 8 to 10 seconds' duration, 10 strokes being applied in all. About 1.5 seconds after the application of the stimulus, the longitudinal muscle started to contract. The height of the contraction was reached about 9 seconds later. The contraction was sustained for about 7.5 seconds, and then relaxed, the base line being reached about 33 seconds after the stimulus was applied.

The circular muscle responded to this *longitudinal* stimulus altogether differently. About 2.3 seconds after the application of the stimulus, there appeared a fall in the lever. This apparent inhibition reached its greatest depth at about 8 seconds, and returned to its base line in 20 seconds from the stimulus.

The question as to whether this depression in the record is a true inhibition, or simply a mechanical effect of the longitudinal contraction, cannot be answered now. The facts that the depression of the circularly applied lever begins approximately 1 second after the longitudinal, and also

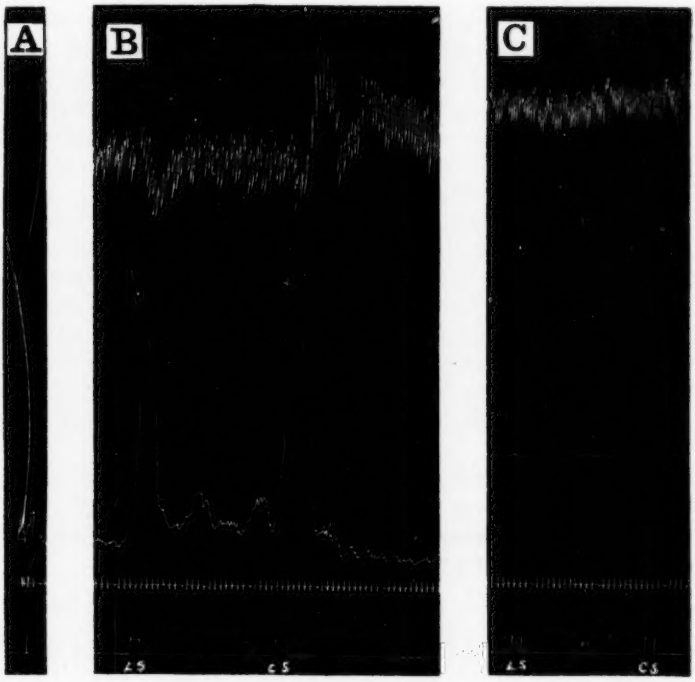

Fig. 3. This tracing of colonic motility of an unanesthetized dog was made with the apparatus shown in figure 2. Time—5 seconds.

A. Shows alignment of the writing levers. Upper tracing is circular movements; lower, longitudinal.

B. *L.S.*—longitudinal stroking between the clips. *C.S.*—transverse stroking between the clips.

The smaller contractions superimposed upon the upper tracing are respiratory.

C. After 1 mgm. of atropine sulphate subcutaneously, the "myenteric reflex" has been abolished. The same is true after cocainization of the mucosa.

reaches its point of greatest depression about 2 seconds before the longitudinal lever has reached its greatest height, suggest that the behavior of the circular lever is a mechanical effect due to the longitudinal contraction. However, occasionally the depression is poor or absent, while, upon observation of many effects, one sometimes sees the circular lever fall before the longitudinal lever has begun to move (see fig. 3, *L.S.*).

c. *Stroke stimulus applied transversely* (fig. 3B). With the clips fastened as in the experiments described above, the stimulus was applied transversely to the long axis of the graft, exactly between the circular clips.

Contraction of the longitudinal muscle occurs after 2.6 seconds with a depression of the circular lever at about 2.8 seconds. The height of the longitudinal lever movement after transverse stimulation averages only 70 per cent of that after longitudinal stimulation. About 7 seconds after the longitudinal contraction is initiated, there is strong and well sustained contraction of the transverse muscle, which lasts several seconds longer than the former. Occasionally there is a secondary contraction of the circular fibres, especially toward the beginning of the experiment, when mucosa seems most irritable (see fig. 3 *C. S.*).

d. Atropine, when given to the animal in doses which accelerate the heart (1 mgm.), abolishes the above reactions (fig. 3C). It seems obvious therefore, that the responses recorded above are reflex in character, and that the application of the stroke stimulus of itself does in no way disturb the relationship of the clips. To test this further, cocaine was applied topically to the mucosa, as a 5 per cent solution, on cotton. The cocainized areas were found to have lost their irritability, for now the muscle did not respond to the stroking. Adjacent areas retained their power to elicit the response.

e. The activity of the two coats of the colon which may be elicited by stimulation of the mucosa *persists after the pedicle of the graft is severed.* This means that the reflex is a local one, and is independent of any connection with the central nervous system.

CONCLUSIONS

1. The motility of the colon musculature has been studied by a new method which allows observation of the motility of each coat of the muscle independently upon stimulation of the exposed colonic mucous membrane.

2. The muscular response of the colon to a stroke stimulus applied to its mucous membrane is reflex in character, for it is abolished by cocaine applied to the mucosa locally, or by atropine administered systemically. This reflex response is independent of the central nervous system, for the reflex persists after the surgical separation of the colon from its original connection with the mesentery.

THE MYENTERIC REFLEX AS EXHIBITED BY THE EXTERIORIZED COLON OF THE DOG

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In 1899 Bayliss and Starling investigated the movements and innervation of the intestine, and concluded, "that if cerebrospinal reflexes be excluded, excitation at any point of the gut excites contraction above, inhibition below. This is the law of the intestine." Since then, many other investigators have confirmed the general truth of this statement, but the fact must be emphasized, that this "myenteric reflex" (Cannon, 1912) cannot be demonstrated invariably, and that it occurs typically only under very special conditions (denervation, purging).

Previous work in this field has been done with intestinal strips (Magnus, 1904) or with anesthetized animals. The changes in motility have been recorded by means of x-rays, balloons put into the bowel through a cut in its wall, or by enterograph of Alvarez (1924). All of these methods cause trauma, although the enterograph method seems to offer the least objection on this score. A more important objection can be found against the mode of stimulation of the bowel. In the researches of Bayliss and Starling, the stimuli were applied either to the serosal surface, or to the mucosa, by means of cotton pledgets or other "bolus-like" material. The bolus stimulus is much better than one applied to the serosa, a surface of no importance in the digestive activity of the intact animal. Distention is to be regarded as the normal mechanical stimulus for both large and small intestine. In studies directed toward localization of action, distention causes a reaction too widespread to be useful.

Alvarez (1924) states that in studying peristaltic activity with "6 or 7 recorders attached to the bowels of rabbits," he was "surprised to find that ordinarily the bowel not only did not relax in front of the advancing wave, but it often became more active, and served to stop the rush, especially in the lower ileum." Alvarez and Starkweather (1919) found that the usual response to *peritoneal* stimulation of rabbit intestines was contraction above and below. The objection to these experiments, which has been duly appreciated by Alvarez (1924), has been that only the motility of the longitudinal coat of the intestine was being recorded. It will be shown

later that this objection holds the key to the situation, and explains many of the discrepancies reported in the literature.

METHOD. The technic used in the present investigation was described in the preceding article. It consists essentially of the exteriorization of a piece of bowel (colon, in the present study) of a dog, so that the mucosal surface is exposed to the air. When the dog has recovered from the operation, he is trained to lie quietly, while the motility of the intestinal transplant is recorded by means of a modified enterograph lever. The longitudinal and transverse musculature may be studied separately or simultaneously, while the chosen stimulus is applied to the mucosa. We have shown already that there is present in the intact graft a reflex which depends upon the adequate sensitivity of the mucosa to a tactile stimulus, since it is abolished by cocaine. The reflex reaches the muscle by way of the para-sympathetic myoneural junctions, for it disappears after atropine. However, the myenteric reflex persists after section of the pedicle which connects the intestinal graft with the central nervous system. The stimulus which was used to elicit the reflex response was applied by stroking the mucosa by means of a dull teasing needle, with as uniform pressure as possible. This stimulus has proved adequate to elicit a reflex response on the part of the intestinal musculature, but does not otherwise disturb the dog.

A few preliminary experiments were performed on pithed cats, with the enterograph attached to the serosal surface of the ileum (Mulinos, 1931).

RESULTS. A. In pithed cats, maintained by artificial respiration, the intestinal motility of which is being recorded graphically, stimulation of the vagus nerve in the neck or chest elicits a strong motor response, which varies in duration with the age of the specimen, the amount of trauma and exposure. It was observed that in about half of over 100 such stimulations the enterogram would indicate a marked relaxation to follow the primary contraction. Of these, about 90 per cent occur when the bowel is dilated initially. Visual examination of the gut during the "relaxation," on the contrary, showed the bowel to be in such severe spasm as to be bloodless. During the contraction of the circular muscle, the intestine between the recording strips was lengthened, giving the appearance of depression or relaxation of the longitudinal muscle, an unwarranted conclusion. These observations led us to refer to further investigation the inference that—

a. The primary contraction recorded was due to a shortening of the longitudinal fibres of the ileum which we were recording, and that

b. The longitudinal contraction was soon *followed* by a contraction of the circular muscle, which was sufficiently potent to overcome the longitudinal shortening, and to lengthen actually these fibres.

Magnus (1904) observed this same phenomenon in isolated strips, and concluded that as the circular coat contracts, the longitudinal relaxes.

Quite probably what Magnus saw was the predominance of the activity of the circular muscle, which, by constricting, stretched the now relaxing longitudinal muscle. Cannon (1912) believed that the two coats contracted simultaneously. From these experiments, and those which follow, it is obvious that after vagus stimulation in the neck, or from stroking

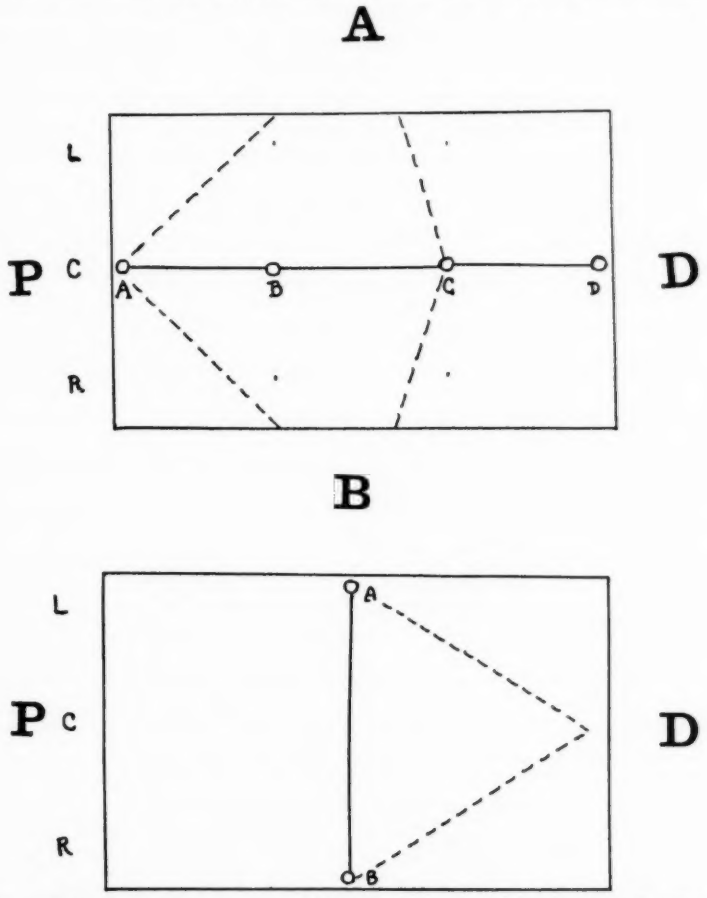

Fig. 1. Schematic representation of the colonic graft. *P* is proximal, *D*—distal; *R*—right and *L*—left. The solid lines ending in circles represent the attachment of the clips of the recording instrument. Further details in text.

A. With the clips attached at B–C, stimuli within the area enclosed by the broken lines are effective in eliciting a contraction of the longitudinal muscle.

B. With the clips at A–B, stimuli within the area of the isosceles triangle of base A–B and the broken lines are effective in eliciting a contraction of the circular muscle between A–B.

the mucous membrane, the longitudinal muscle contracts *first*, and is *followed* by a contraction of the circular coat.

B. *Experiments on the unanesthetized dog.* 1. Localization of the position of the stimulus necessary to produce contraction of the longitudinal muscle. With the clips of the recording apparatus attached at any one location, contraction of the muscle recorded by the clips does not follow stimulation of all parts of the mucosa of the graft. The points stimulated are best shown with the aid of a graph, figure 1, a. The circles indicate the position of attachment of the recording clips from time to time while the lines indicate the position of stimulus application. The letters *A*, *B*, *C*, *D* indicate the position of the recording clips, while *C*, *L*, *R* indicate the center, left and right of the graft.

With the clips at *A-B*, the stimulus was applied at the various points shown in the graph. The results are shown in the accompanying tables.

Recording clips attached at A–B (fig. 1-A)

STIMULUS POSITION	EFFECT	STIMULUS POSITION	EFFECT	STIMULUS POSITION	EFFECT
AB–L	Moderate contraction	BC–L	No contraction	CD–L	No contraction
AB–C	Strong contraction	BC–C	No contraction	CD–C	No contraction
AB–R	Moderate contraction	BC–R	No contraction	CD–R	No contraction

Recording clips attached at B–C (fig. 1-A)

STIMULUS POSITION	EFFECT	STIMULUS POSITION	EFFECT	STIMULUS POSITION	EFFECT
AB–L	Moderate contraction	BC–L	Moderate contraction	CD–L	No contraction
AB–C	Strong contraction	BC–C	Strong contraction	CD–C	No contraction
AB–R	Moderate contraction	BC–R	Moderate contraction	CD–R	No contraction

Recording clips attached at B–D (fig. 1-A)

STIMULUS POSITION	EFFECT	STIMULUS POSITION	EFFECT	STIMULUS POSITION	EFFECT
AB–L	Weak contraction	BC–L	Moderate contraction	CD–L	Moderate contraction
AB–C	Moderate contraction	BC–C	Strong contraction	CD–C	Strong contraction
AB–R	Weak contraction	BC–R	Moderate contraction	CD–R	Moderate contraction

It is obvious that a stimulus applied to the colonic mucosa "below" that is distal to the point which is being recorded, elicits no contraction

of the longitudinal muscle above. Stimulation of the mucosa above or orad to the point of record elicits strong contractions at and below the application of the stimulus.

These three tables show that stimuli applied along the same longitudinal plane as the recording clips will produce contraction only if they are applied between the clips or proximal to them. When the stimulus is applied lateral to the clips the motor response diminishes rather rapidly. Applied proximally, the stimulus is effective over a distance of 4 to 6 cm. Applied laterally, it is effective only within 2 to 3 cm. This reflex reaction becomes intelligible upon the assumption that there are nerve connections which run sensory-motor distal to the point stimulated. *Apparently the mucosa has no nervous connection with the longitudinal muscle above itself.*

SUMMARY. Stimulation of the mucosa of the colon causes a contraction of the longitudinal muscle at and below the point of stimulation, but none above. With a linear stimulus, the area of contraction may be represented by an isosceles triangle. Conversely, a given area of the intestine contracts when stimuli are applied to the mucosa above the area. Such a condition acting alone would cause an increase in the volume of the intestinal lumen below the point of stimulation (bolus).

2. Localization of the position of the stimulus necessary to produce contraction of the circular muscle. The diagram (fig. 1b) shows the position of the clips on the graft, and the points stimulated. The clips were attached to A–B. The stimuli were applied transversely, between the clips, and at 2 and 4 cm., on either side. The results are shown in the accompanying table.

Recording clips attached at A–B (fig. 1-B)

STIMULUS POSITION	LEFT	CENTER	RIGHT
Proximal to clips 4 cm...	No contraction	No contraction	No contraction
Proximal to clips 2 cm...	No contraction	No contraction	No contraction
Between the clips...	Strong contraction	Strong contraction	Strong contraction
Distal to the clips 2 cm...	Weak contraction	Strong contraction	Weak contraction
Distal to the clips 4 cm...	No contraction	Weak contraction	No contraction

It is obvious from these results that the circular muscle responds to stimuli in a manner different than does the longitudinal. All stimuli proximal to the clips are incapable of eliciting a contraction of the circular muscle between the clips. Stimulation of the colonic mucosa at and distal to the recording clips elicits a response. When the stroke stimuli are applied transversely, both the circular and the longitudinal muscles respond by contraction. It may be assumed that there are nerve connections which

run sensory-motor proximal to the point stimulated. *The mucosa appears to have no nervous connections with the circular muscle below itself.*

SUMMARY. Stimulation of the mucosa of the colon causes a contraction of the circular muscle at and above the point of stimulation, but none below. With a linear stimulus applied transversely, the area of contraction may be represented as an isosceles triangle (fig. 1b). Conversely, a given strip of the intestine contracts to stimuli applied to the mucosa below the strip. Such a condition acting alone would prevent easy access of a bolus toward the stomach; acting together with the longitudinal muscle, there will be an increase in lumen area below the stimulus, a decrease above.

DISCUSSION. The results described in the preceding paper, and under A in the first part of this report show beyond any shadow of doubt that vagus stimulation, or mucosal irritation, *elicit contractions of the longitudinal muscle first, and then of the circular.* The result of such a mechanism is first to increase the lumen of the gut, and then to decrease or obliterate it. These experiments offer no suggestion that either coat of the colonic musculature is inhibited at any time by mucosal stimulation. If a balloon system is used as a recorder, the effect—longitudinal contraction below, followed by circular spasm above—may be interpreted as inhibition (increased volume of intestinal lumen), followed by stimulation. When the enterograph system is used (Mulinos, 1931) what is recorded is an apparent stimulation (contraction of the longitudinal muscle), followed by relaxation.

The results obtained by a more careful localization of the relationship between the locus of stimulation and the region of response point definitely to the existence of a "myenteric reflex." The sequence of events is something like this: A stimulus (bolus) at any point along the colon elicits a contraction of the longitudinal muscle at and below the point of stimulation. The intestinal lumen below the bolus is enlarged, facilitating its passage downward. A few seconds *later*, there is contraction of the circular muscle at and above the point of stimulation. The intestinal lumen above the bolus is narrowed or obliterated, further facilitating the downward passage of the bolus.

This phenomenon is a true reflex, for it is abolished both by cocainization of the mucosa, and by atropine sulphate given systemically. It is a true myenteric (local) reflex for it does not depend upon any connection with the spinal cord. All that can be said at this time is that this myenteric reflex occurs in intact, unanesthetized dogs. No denervation or purging is necessary (Bayliss and Starling, Cannon, Alvarez). It is possible that the localized stimulus studied, if continued downward along the colonic mucosa, might result in a "chain" reflex, giving the appearance of peristalsis. No such studies were made.

It has been mentioned already that the mucosa is connected functionally

with the two muscular coats of the colon in a very characteristic manner. Sensory nerves (afferent) connect the mucosa with the longitudinal muscle at and below itself; and with the circular muscle at and above. Furthermore, impulses to the longitudinal muscle become manifest more quickly than impulses to the circular muscle. It is impossible at this time to say whether the difference in rate of response is to be found in the afferent fibers of the nerves, or in the circularly arranged muscle fibers. It may be that in the case of the longitudinal muscle fibers the afferent nerves have their endings, and presumably the source of acetyl-choline or other such hormone (Mulinos, 1929), very near to the parasympathetic myo-neural junctions of the muscle. The quickness of response would be then a function of the rate of diffusion of the "vagus-stoffe" into the cell. Contrariwise, the smooth muscle cells of the circular muscle may lie at a greater distance from the nerve endings, giving a response which is slower than that of the longitudinal muscle by the time necessary to diffuse into the cell. This same delay in transit opens the acetyl-choline to the destructive action of the esterase, so that less of the drug reaches the cell. Such a concept may explain the observation that longitudinal stimulation elicits a response only in the longitudinal muscle, while transverse stimulation elicits contractions from both the coats. We may picture the intestinal mucosa as made up of a mosaic of sensory nerve receptors. These are arranged in rows of pairs, one member of each pair to the circular and the other to the longitudinal muscles. Upon longitudinal stimulation, we stimulate a large number of pairs, but only a few of those which run to any one set of circular muscle fibers. The very little acetyl-choline which is liberated near the circular muscle is destroyed by its esterase before it can diffuse into the cell, and there is no contraction. When the stimulus is applied transversely there is much more response because the impulses to the muscle are manifold those from longitudinal stroking. The longitudinal muscle fibers respond both times because they are situated nearer their corresponding nerve endings. We speak of this as greater sensitivity for want of a better term. We believe that the sensory elements of the myenteric reflex have their cell stations within the gut wall because the myenteric reflex persists after the graft has been isolated from the central nervous system.

One observation deserves mention at this time. After food, the colonic graft seemed to be more irritable, so that a lesser mucosal scratch elicited a response, while the normal stimulus elicited more powerful and longer sustained contractions of the intestinal coats. A plausible explanation is offered of the fact that eating of food (distention of the stomach) causes an increased desire to stool, and that this increase in colonic activity is not dependent upon an increase of the contents of the colon. White, et al. (1934) report a reddening of, and an increased secretion by the mucous membrane of the colon in man immediately on beginning to eat.

SUMMARY

1. The motility of the colon graft elsewhere described was studied on unanesthetized trained dogs. The stimulus is applied to the mucosa.

2. The principle of the "myenteric reflex" has been confirmed, although a new definition became necessary.

3. Our results point definitely to the absence of any inhibition in the make-up of the myenteric reflex—and also demonstrate that no such mechanism is necessary for the propulsion of a bolus along the colon.

4. Thus "the law of the intestine" may be restated as follows: In the colon of the unanesthetized dog, *a stimulus applied to the mucosa is followed by contraction of the longitudinal muscle at and below the stimulus, and by contraction of the circular muscle at and above it. The first precedes the latter by 3 to 5 seconds.*

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THE INTRINSIC REGULATION OF THE CIRCULATION IN THE HYPOTHALAMUS OF THE CAT

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In an earlier publication (Schmidt and Pierson, 1934) a report was made of an investigation of the circulation in the medulla of the cat by means of a small, artificially cooled thermocouple inserted directly into the tissue. The method, an adaptation of that used by Gibbs (1933), is applicable to any part of the central nervous system, and it is our purpose to use it in a study of the circulation in various parts of the brain. The hypothalamus was chosen as the second region for study because our interest was not confined to the vascular regulation *per se* but included the relation of intrinsic vascular phenomena to the regulation of the activity of vegetative centers, and the hypothalamus ranks a close second to the medulla in the distribution of vegetative centers.

The methods used in this investigation were the same as those described in the earlier paper (Schmidt and Pierson, 1934). The same thermocouple was used, with ice-water in the thermos flask. Access to the hypothalamus was obtained by dissection through the pharynx from above, with the animal on its back. After the base of the skull was exposed, a 5 mm. hole was made through the bone with a hand drill, with utmost pains to avoid perforation of the dura. The landmark for the hole, determined on sectioned heads, was the intersection of the midline with a line joining the anterior borders of the auditory bullae. This was the posterior (caudal) margin of the drilled hole. A hard-rubber guide of suitable length and thickness was screwed into the hole; a thread cut on the lower end of the guide, and a corresponding thread cut into the bone by a steel tap, insured rigidity. The thermocouple was inserted through this guide, and through the previously unopened dura, with sufficient pressure to force the rubber stopper of the thermos flask around the free end of the guide, thus forming a water-tight seal. The flask was then clamped in the vertical position.

Cats were used exclusively. They were narcotized by an intraperitoneal injection of sodium amytal (0.06 gram per kilo) or sodium pentobarbital (0.032 gram per kilo). In all of the experiments upon which this

report is based they were also given curara (7–10 mgm. per kilo) intravenously and were under artificial respiration supplied by a double Meyer pump. Blood-pressure was recorded from a femoral artery by a mercury manometer; the anticoagulant was sodium thiosulfate (25 per cent). A femoral vein was used for intravenous injections. For electrical stimulation faradic current was used; it was supplied by a Harvard inductorium furnished with two dry-cells; an open platinum electrode was employed.

Control procedures were the same as those used in our experiments on the medulla (Schmidt and Pierson, 1934), excepting perfusion of excised heads, repetition of which was deemed unnecessary. Abolition of the heat gradient in the thermocouple, by substitution of water at body temperature for the ice-water with which the thermos flask was ordinarily filled, did not yield as clean-cut results with the instrument in the hypothalamus as it did in the previous experiments on the medulla. Whereas the galvanometric deflections in response to adrenalin, carotid occlusion, asphyxia, etc., were uniformly absent when the instrument was inserted without heat gradient in the medulla, the corresponding tests in the hypothalamus showed some deflections; they were, however, always much smaller, much more sluggish, and much less reversible than they were with ice-water in the flask. We believe this to indicate that the thermal insulation of the exposed hypothalamic region was less perfect than that of the medulla, so that some heat gradient remained even when the flask was filled with water at body temperature. The galvanometric deflections were so uniformly increased in extent, rapidity, and reversibility by restoration of the ice-water heat gradient that we feel justified in attributing them to changes in blood-flow, not in heat production.

An additional set of control experiments was made to test the capacity of CO_2 *per se* to set up an electromotive force in the thermal junction. For this purpose the instrument, prepared as usual with ice-water in the flask, was inserted through the guide usually employed into a stream of distilled water passing the thermal junction at a measured constant rate. Three solutions were used: 1, ordinary stock distilled water, containing 0.47 vol. per cent of CO_2, with pH 6.8 to phenol red; 2, boiled and cooled distilled water, containing 0.29 vol. per cent CO_2, with pH 7.1; 3, distilled water saturated at room temperature with pure CO_2 under pressure, containing 67 vols. per cent CO_2 and with pH lower than 6 (the lower limit of the indicators used). All three solution reservoirs, the thermal junction, and all the tubing through which the solutions flowed, were immersed in water in a large tank. Two experiments were made with simple gravity perfusion, one at 40°C., the other at room temperature. A third was made with perfusion of the solutions at room temperature, by means of a pump. In all three experiments we found that substitution of the CO_2-saturated solution for a solution nearly free of CO_2 had no consistent or definite

effect upon the position of the galvanometer when perfusion flow remained constant. Since the CO_2 contents of these solutions (0.29, 0.47, and 67 vols. per cent) afforded a much wider range than any that could conceivably be encountered in living tissues, we conclude that the galvanometric deflections produced by CO_2 insufflation in our experiments on the medulla and hypothalamus were actually due to changes in blood-flow, and not to a direct influence of CO_2 upon the thermal junction.

In order to determine the position of the instrument, at the end of each experiment we severed the animal's head and split it longitudinally in the midline with a saw. In most cases the antero-posterior position had been properly fixed (i.e., between the lips of the sella turcica), but the junction was often not exactly in the midline (determined by the position of the basilar artery). The deviation was usually about 2 mm. or less, but in one experiment it was 4 mm. This seeming error in technic proved to be fortunate because it enabled us to determine that the constrictor effect of cervical sympathetic stimulation upon hypothalamic blood-vessels is essentially ipsilateral. Furthermore, the pituitary body was not always in the path of the thermocouple. Penetration of the tip of the thermocouple into the substance of the hypothalamus was from 2 to 4 mm. There was often some bleeding from the bone when the hole was drilled, but this stopped promptly and clots were usually not found inside the skull at the autopsy.

I. Vasomotor innervation. Faradic stimulation was applied to the cervical sympathetic (uncut, cephalic and thoracic ends after cutting), vagodepressor (central and peripheral ends), and carotid sinus nerves, and to the exposed carotid sinuses directly. The stimulations varied from 5 seconds to 3 minutes in duration, and from 12 cm. (distance of secondary from primary) 85° (angle of secondary from horizontal) to 2 cm. 0° in intensity. The results of stimulation of the vagodepressor and carotid sinus mechanisms may be dismissed with the statement that they were entirely negative from the standpoint of active changes in circulation in the hypothalamus. Indicated blood-flow followed passively a change in blood-pressure when it occurred, while otherwise flow was unaffected. Central vagus stimulation was tried repeatedly in 25 cats, and sinus stimulation in 14; all of these animals showed definite sensitivity to the dilator effects of CO_2 and most of them responded with vasoconstriction to stimulation of a cervical sympathetic nerve. In four additional experiments artificial respiration was used without curarization, the narcotic being amytal; the results were equally negative. We conclude that the vagodepressor and carotid sinus systems do not contain vasodilator fibers that are effective in the hypothalamus of the cat narcotized with amytal or pentobarbital. This assumes, of course, that such fibers would be thrown into activity by faradic stimulation.

Cervical sympathetic stimulation, however, produced consistent and sometimes quite marked reduction in hypothalamic blood-flow. The effect was not a passive one for it occurred without fall, or with an actual rise in blood-pressure. There were no muscular movements during the stimulation, since the animals were curarized; pulmonary ventilation and body (rectal) temperature were unchanged. The conclusion is therefore justified that hypothalamic vessels were constricted as a result of faradic stimulation of the uncut cervical sympathetic nerve, or of the cephalic end of the cut nerve (fig. 1).

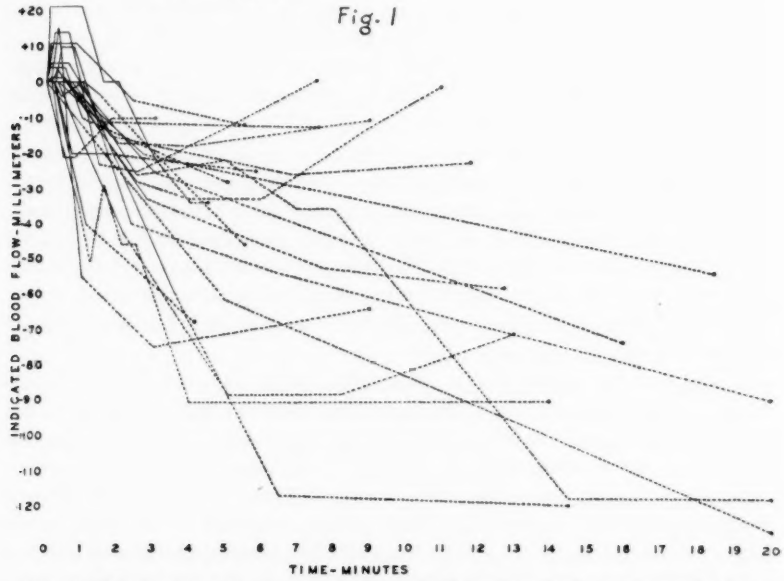


Fig. 1. Effect of faradic stimulation of cervical sympathetic nerve upon blood-flow in hypothalamus.

From 29 experiments on curarized cats narcotized with amytal or pentobarbital. Explanation in text. Each curve is from a different experiment.

The data upon which this conclusion is based were obtained in 39 experiments on curarized animals in which the recording instrument showed good sensitivity to carotid occlusion, CO_2 insufflation, and intravenous injection of adrenalin. Cervical sympathetic stimulation produced clean-cut reduction in hypothalamic blood-flow, without fall or with a rise in blood-pressure, in 29, or about 74 per cent. Of these animals 23 were narcotized with amytal, 16 with pentobarbital; the distribution of positive results was identical, i.e., 17 of the animals under amytal (74 per cent), 12 of those under pentobarbital (75 per cent).

There were several noteworthy features in this hypothalamic vasoconstriction following cervical sympathetic stimulation: First, relatively strong currents were necessary; usually the secondary coil had to be horizontal and 12 cm. or less from the primary to elicit positive results. Second, there was practically always a definite latent period during which hypothalamic blood-flow either rose or showed no change; this period ranged from two seconds to one minute, averaging 25.2 seconds. Third, the vasoconstriction, once initiated, progressed after the stimulation in all but 2 animals; the maximum effect was attained in one experiment only at 45 minutes after the stimulation; the average time was 3.1 minutes. Fourth, recovery from the vasoconstrictor effect was complete in only 2 animals (7 per cent); it was partial (including any tendency in that direction) in 7 others (24 per cent); in the remaining 20 animals (69 per cent) there was no trace of recovery over a period ranging from 6 to 45 minutes after the stimulation.

These results are portrayed graphically in figure 1. Only the positive results (i.e., reduction in hypothalamic blood-flow without a fall or with a rise in blood-pressure) are shown. In order to distinguish the period of stimulation, it is shown as a solid line; the recovery period is represented by the broken continuation. Each curve represents the maximum positive effect of cervical sympathetic stimulation in one experiment. A large number of cases in which there were marked and promptly reversible decreases in hypothalamic blood-flow are not included because blood-pressure fell during the stimulation and recovered promptly afterward; these effects may not have been entirely passive, but there is no way to distinguish actual vasoconstriction from the passive effect of change in blood-pressure. The period of observation shown in these curves represents only the time during which there was no fall in blood-pressure after the stimulation.

Two additional points, not shown in the curves, deserve mention: First, after an effective stimulation repetition even with much stronger current usually was ineffective. Second, the vasoconstrictor response was essentially an ipsilateral one: in 20 of the 29 positive experiments the thermal junction was found to have been somewhat (1 to 4 mm.) to one side of the midline, and in 14 of them (70 per cent) there was hypothalamic vasoconstriction only on stimulation of the ipsilateral nerve; in 3 (15 per cent) there was some response to stimulation of either nerve, and in the remaining 3 the response was only contralateral. In the 9 experiments in which the instrument was in the midline there was hypothalamic vasoconstriction on stimulating either nerve in 6; in 2 there was constriction on stimulation of the right nerve, dilatation on stimulation of the left; in the remaining one stimulation of the left nerve caused constriction while the right had no effect.

Some of the above-mentioned features in the response of hypothal-

amic blood-flow to stimulation of the cervical sympathetic nerve seemed sufficiently unusual to warrant further consideration. This is particularly true of the latent period, the slow progression, and the tendency to irreversibility and lack of repeatability. It is possible that these are characteristic of the recording instrument or of the mode of stimulation and not of the vasoconstrictor innervation of hypothalamic blood-vessels. This point was investigated in 7 experiments on curarized cats narcotized with amytal (3) or pentobarbital, in which the instrument was inserted in

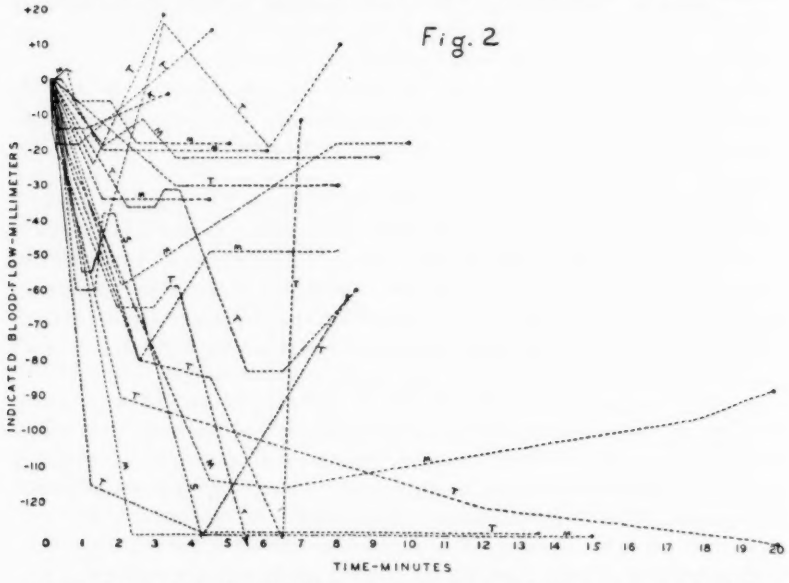


Fig. 2. Effect of faradic stimulation of cervical sympathetic nerve upon blood-flow in temporal muscle or tongue.

From 7 experiments on curarized cats narcotized with pentobarbital. Arrangement same as figure 1. Repeated stimulations were often effective and such as were effective are included; the curves are not all from different experiments.

a temporal muscle or lateral to the midline of the tongue; in one experiment it was also inserted in the kidney and spleen. The results of stimulation of the ipsilateral intact or cut cervical sympathetic nerve (or of the uncut splanchnic nerve in the case of the kidney and spleen) are shown in figure 2, which is constructed in the same manner as figure 1: the solid lines represent the period of stimulation, the broken lines recovery. The letter *M* indicates temporal muscle, *T* tongue, *K* kidney, and *S* spleen.

It is evident at once that the latent period that was so characteristic of the response of hypothalamic blood-flow was not present in muscle, tongue,

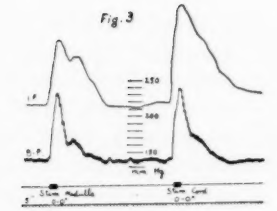
spleen, or kidney. Furthermore, the vasoconstriction attained its maximum much more rapidly in the extracranial tissues. The appearance is that of a much more intense vasoconstriction in the latter, but since the recording instrument is inherently not a quantitative one, this point is only suggested. Evidently a long latent period and a slowly progressing response are not characteristics of the recording device, of the cervical sympathetic nerve, or of the type of nerve excitation employed in these experiments.

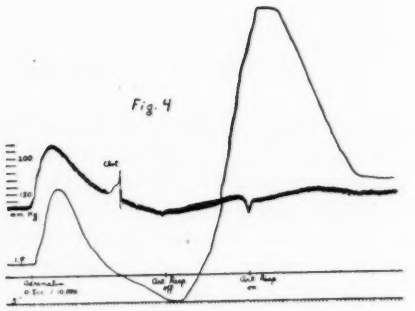
With regard to the reversibility of the vasoconstrictor effect in temporal muscle or tongue, there was decidedly more tendency in that direction than there was in the hypothalamus, but in some cases the effect was irreversible here also. This was apt to be the case when we used strong currents, comparable with those required to elicit any response in the hypothalamus; with weaker currents (i.e., 12 cm. 60° to 12 cm. 30°) the vasoconstriction in temporal muscle or tongue was usually completely and promptly reversible. It is probable therefore that the irreversibility of the hypothalamic vasoconstriction was due to the strong currents employed, but by the weaker currents hypothalamic blood-flow was not affected at all. Duration of stimulation seemed to have no great bearing on intensity or reversibility of the vasoconstrictor response in temporal muscle, tongue, or hypothalamus, provided only that the stimulation was continued until vasoconstriction began.

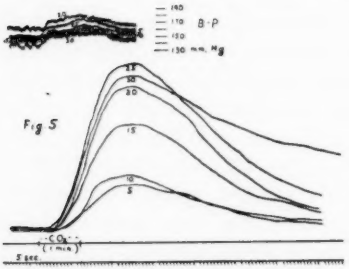
It is noteworthy that vasoconstriction could be produced repeatedly in the muscle or tongue, even without increasing the strength of the current applied to the cervical sympathetic nerve. This makes it unlikely that the lack of repeatability encountered in the hypothalamus was due only to damage to the nerve by the manipulations used in stimulating it. It seems much more probable that persistence of maximal vasoconstriction was responsible. This constriction could be temporarily overcome by asphyxia or CO_2, or by intracarotid injection of histamine or a choline derivative, and while any of these vasodilator agents was acting cervical sympathetic stimulation was more effective than ever.

Another point of considerable importance is that blood-flow in the muscle or tongue increased very definitely and consistently when both cervical sympathetic nerves were cut; in the hypothalamus section of both cervical sympathetics never caused any increase in blood-flow.

Because of this last observation, together with the longer latent period, slower progression, and poorer repeatability and reversibility of the hypothalamic response, it seemed possible that the cervical sympathetics are not a normal pathway for vasoconstrictor impulses to the hypothalamus. The results shown in figure 1 leave no doubt concerning a vasoconstrictor innervation that can be activated by strong and prolonged stimulation of this nerve, but it seemed possible that the results represented a more or







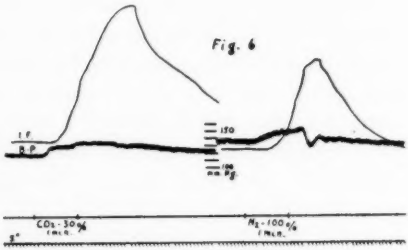


Fig. 3. Faradic stimulation of brain-stem: effect on blood-pressure and hypothalamic blood-flow.

February 14, 1934. Cat: amytal and curara, artificial respiration. Faradic stimulation with secondary at 0 cm. from primary. First stimulation: one wire in medulla, other on needle inserted under mucous membrane of mouth. Second stimulation: wire removed from mouth and attached to another wire previously inserted in lower thoracic cord, other terminal remaining in medulla. Records from above downward: Indicated blood-flow in hypothalamus (*I.F.*); femoral blood-pressure (*B-P*) with calibration (mm. Hg); signal; time in 5 second intervals, with a break at one minute.

All records to be read from left to right. To insure accurate reproduction at the size necessitated by space restrictions, the original smoked-paper records have been copied over a transparent plate.

Fig. 4. Effect of adrenalin and asphyxia.

February 7, 1934. Cat: amytal and curara, artificial respiration. Arrangement same as figure 3; calibration (mm. Hg) applies to blood-pressure, but can be used for indicated blood-flow (*I.F.*) if multiplied by 2.

Adrenalin injected intravenously.

Artificial respiration discontinued between signals, until blood-pressure began to fall. There were no respiratory movements.

Fig. 5. Effect of ascending blood CO_2 content.

March 19, 1934. Cat: pentobarbital and curara, artificial respiration. CO_2 5, 10, 15, 20, 25, and 30 per cent in air aspired through respiration pump for one minute. Blood-pressure changes are superimposed at upper left, with calibration; the rises were 10 mm. with the 5 per cent mixture, 10 mm. with 10 per cent, 26 mm. with 15 per cent, 20 mm. with 20 per cent, 26 mm. with 25 per cent, and 18 mm. with 30 per cent.

Fig. 6. Maximal effect of CO_2-excess and O_2 lack.

April 19, 1934. Cat: pentobarbital and curara, artificial respiration. CO_2, 30 per cent in air (rise in blood-pressure 20 mm.); pure nitrogen (rise in blood-pressure 12 mm.), each for one minute via respiration pump. Nitrogen insufflation 8 minutes after CO_2.

less accidental impingement of a highly artificial stimulus upon a more direct vasomotor nerve pathway. In order to test for the presence of an intracranial vasoconstrictor nerve system involving hypothalamic vessels, we resorted to direct faradic stimulation of the brain-stem. One secondary wire was passed into the medulla through a cisternal puncture needle; the other wire was connected to a needle inserted under the mucous

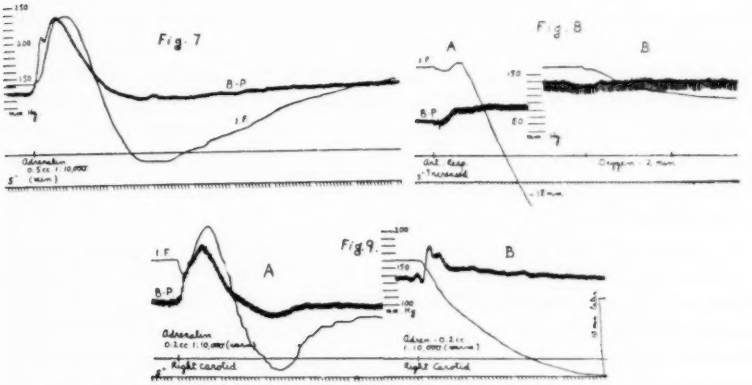


Fig. 7. Effect of adrenalin (intravenous injection).

February 17, 1934. Cat: amytal and curara, artificial respiration. Injection into femoral vein; Parke, Davis & Co. solution, diluted 1:10 with saline.

Fig. 8. Effect of increased ventilation and of oxygen.

A. From same experiment as figure 4. At signal thrust of respiration pump was increased, rate remaining unchanged. Blood-pressure rose from 84 to 106; indicated flow fell to a point 18 mm. below bottom level of tracing—a total of 118 mm.

B. March 8, 1934. Cat: pentobarbital and curara, artificial respiration. Oxygen via respiration pump for 2 minutes between signals. Blood-pressure rose from 134 to 138. Indicated flow fell 21 mm.

Fig. 9. Intracarotid injection of adrenalin: effects on blood-flow in hypothalamus and temporal muscle.

A. June 12, 1934. Cat: pentobarbital and curara, artificial respiration. Instrument in hypothalamus, 1 mm. to right of midline. Indicated flow recovered completely within 5 minutes after end of this record.

B. June 13, 1934. Cat: pentobarbital and curara, artificial respiration. Instrument in right temporal muscle. Indicated flow recovered partially as shown within 10 minutes after this record.

membrane of the roof of the mouth. The recording instrument was inserted into the hypothalamus as usual; the narcotic was pentobarbital and the animals were curarized. Six experiments were made. The results were as follows:

No valid evidence was obtained of a hypothalamic vasoconstrictor innervation that could be activated by faradic stimulation of the brain-stem

in this manner; although strong excitation of vasomotor mechanisms was evident in marked hypertension during the stimulation, hypothalamic blood-flow simply followed the blood-pressure change (fig. 3). Currents of varying strength were used, but the only effects on hypothalamic blood-flow were apparently passive. When the wire was removed from the mouth and inserted into the lower thoracic cord, so that the stimulation involved the medulla and the cervical and thoracic regions of the cord, the blood-pressure response was somewhat greater than before, and so was the effect upon hypothalamic blood-flow (fig. 3). As a control on these responses, the stimulations were repeated after the animal had died: the recording instrument indicated no change. We also attempted to utilize the silver tip of the thermocouple as one terminal for stimulation, instead of the wire in the mouth or thoracic cord, but the results are useless because stimulation after the animal was dead still produced deflections of the galvanometer.

It is thus evident that we have been unable to detect a potent intracranial vasoconstrictor innervation to hypothalamic vessels. The long latent period, slow progression, and poor reversibility of the hypothalamic vasoconstriction in response to cervical sympathetic stimulation must therefore, at least for the present, be regarded as characteristics of the response of these vessels to the only vasoconstrictor innervation that we have been able to discover. These characteristics suggest a humoral mechanism—a liberation of a chemical vasoconstrictor agent by the stimulation. In some cases in the hypothalamus (fig. 1) and more frequently in the temporal muscle or tongue (fig. 2) the response was a diphasic one—an initial constriction with partial recovery, followed by a second more marked and irreversible constriction. A humoral mechanism, acting after the direct nerve influence had begun to wear away, is a possible explanation. We have made some observations along these lines; they appear in the following section of this paper.

II. Chemical influences. In these experiments, as in the similar ones on the medulla (Schmidt and Pierson, 1934), our attention was centered upon agencies which might be concerned in the normal intrinsic regulation of the circulation in the hypothalamus. These were CO_2, oxygen, acid and alkali; in addition adrenalin, pituitrin, calcium, histamine, and a choline derivative were tested.

1. *Changes in CO_2, oxygen, and pH of arterial blood.* The procedures were identical with those used in the corresponding experiments in the medulla, excepting the use of pentobarbital as the narcotic in much of the present work. In some experiments amytal was used, and the results were not perceptibly different. All of the animals were curarized.

The results were entirely analogous to those previously reported for the medulla. Asphyxia (cessation of artificial respiration), CO_2 excess, or

oxygen-lack all caused hypothalamic blood-flow to increase consistently and regularly. When blood CO_2 was increased progressively, the blood-flow also increased in a step-like progression (fig. 5). The same was true of oxygen-lack (nitrogen insufflation). The maximal effect of CO_2 was always greater than the maximal effect of anoxemia (fig. 6). The threshold to CO_2 was in the neighborhood of a change of one volume per cent in arterial CO_2; that to oxygen-lack was in the neighborhood of a blood oxygen content of 12 volumes per cent. Overventilation frequently caused reduction in hypothalamic blood-flow even when blood-pressure did not fall or actually rose; oxygen inhalation acted similarly, but much less powerfully or consistently (fig. 8).

Examples which illustrate these reactions are shown in figures 4, 5, 6, and 8. Because the results are so similar to those previously reported in the medulla, more detailed presentation seems unnecessary. It is necessary however to point out certain respects in which the responses of hypothalamic vessels to these chemical agents differed from those of the medulla.

In the first place, the dilator effect of CO_2 on hypothalamic vessels seemed to have an upper limit that was not encountered in the medulla. The effect of insufflation of 30 per cent CO_2 in air was usually little if at all greater than that of 20 or 25 per cent mixtures, although blood CO_2 varied with the aspired gas mixture. In the medulla, the dilator effect of a 30 per cent mixture was always proportionately greater than that of a 20 or 25 per cent mixture. This may mean that vasomotor mechanisms antagonize the effect of CO_2 upon hypothalamic vessels; we are more inclined to believe that the capacity of hypothalamic vessels to dilate is less than that of medullary vessels under comparable conditions, possibly because the initial tone of the latter is greater than that of the former.

Second, we have never seen any definite signs of dilatation of hypothalamic vessels as a result of absorption of fixed acid although blood pH was reduced by as much as 0.25. In the medulla there were occasional instances of vasodilatation under similar circumstances. In neither location were we able to detect vasoconstriction as a result of absorption of alkali.

With these differences, which are probably of minor importance, the response of hypothalamic vessels to changes in CO_2 and oxygen content of the blood were essentially the same as those of medullary vessels of similar animals under comparable conditions.

2. *Adrenalin, pituitrin, calcium, histamine, and a choline derivative.* These agents were tested because of their possible significance to the intrinsic regulation of the hypothalamic circulation. The characteristics of the response of hypothalamic vessels to sympathetic nerve stimulation were such as to suggest the intervention of a humoral mechanism, and

adrenalin (or a substance acting similarly) was an obvious selection for testing. Pituitrin possessed a peculiar interest because of the proximity of the pituitary body to the area under investigation. Calcium was tried because of the possibility that it may be concerned in the regulation of certain hypothalamic functions, and a vascular effect seemed worthy of consideration. Histamine and a member of the choline group were used as representatives of vasodilator agents which might be concerned in intrinsic vascular control.

The substances were first tested by intravenous injection in curarized cats narcotized with pentobarbital. Pituitrin and calcium gave completely negative results: hypothalamic blood-flow followed passively any change in blood-pressure that occurred, and was unaffected when blood-pressure was unchanged. Adrenalin, which was used as a routine test of sensitivity of the preparations, always caused a pure increase in hypothalamic blood-flow as blood-pressure rose, but there was often a period of definite decrease in blood-flow as pressure came down to normal, with slow recovery (figs. 4 and 7). We never observed comparable effects from adrenalin in the medulla. A relatively weak but rather prolonged constriction of hypothalamic blood-vessels by adrenalin is strongly suggested. Histamine and the choline derivative (ethyl ether of B methylcholine) injected intravenously, caused definite increases in hypothalamic blood-flow, but the increases were more or less completely interrupted by the fall in blood-pressure.

In order to bring out the direct effects of these substances upon hypothalamic vessels, they were injected headward into a carotid artery. Pains were taken to have the injected fluid at body temperature, and the quantity of fluid (0.2 cc.) was so small that blank injections of warm saline had no effect. The results in 6 experiments were as follows:

Adrenalin (1:10,000 dilution) usually caused an initial fall in hypothalamic blood-flow, but the fall was always replaced by a rise when blood-pressure subsequently rose from the systemic action of the drug (fig. 9A).

Pituitrin (1:10 dilution) never caused even a trace of reduction in hypothalamic blood-flow at any time. The same is true of calcium (10 per cent).

Histamine (1:100,000 dilution) had effects similar to those of an intravenous injection, but usually much more marked. The dilator effect was immediate in onset, interrupted by the subsequent fall in blood-pressure, then resumed as pressure began to rise; it wore away within a few minutes in the face of a constantly rising blood-pressure.

Ethyl ether of B methylcholine (1:10,000 dilution) acted similarly to histamine, but more rapidly and less persistently.

To insure immediate access of the injected fluid to the hypothalamus, the intracarotid injections were repeated with the opposite carotid closed.

The results were no different from those obtained with the opposite carotid open. The injections were made alternately into both carotids, without significant variations in the results. Apparently the hypothalamic region receives blood about equally from both carotids, as its anatomical relations suggest.

These results justify the exclusion of pituitrin and calcium as agents which affect hypothalamic blood-vessels directly and powerfully. The effects of adrenalin in favorable instances are fully comparable with those of sympathetic nerve stimulation, i.e., adrenalin seems to cause a relatively weak constriction of hypothalamic vessels, readily overcome by the rise in blood-pressure consequent upon more intense vasoconstriction elsewhere, but more persistent than the constriction in other parts of the body. Histamine seems to be at least equal to CO_2 as a dilator of hypothalamic vessels; in some instances histamine was decidedly more powerful than CO_2 in this respect. We compared the response of the instrument in the hypothalamus with that elicited from the temporal muscle when the same dose of histamine was injected into the carotid of the same side; the response of the muscle was decidedly greater and more lasting. The same is true of adrenalin, intracarotid injection of which produced pure vasoconstriction in the muscle (fig. 9B).

We have made a number of incidental observations of other drug effects that deserve mention. Morphine, which we found to cause marked dilatation of medullary blood-vessels, had no definite effect on hypothalamic blood-flow. Ether caused an increase here as it did in the medulla. So did amyl nitrite. Ergotamine, given to the point of marked reduction in the adrenalin effect (3 mgm. per kilo—complete "reversal" did not occur) caused a rise in blood-pressure associated with a pure increase in hypothalamic blood-flow. Sodium bromide, given intravenously in total dosage of 1 gram in a cat showing irreversible constriction of hypothalamic vessels after cervical sympathetic stimulation, had no definite effect upon the hypothalamic circulation. Ephedrine caused pure and probably entirely passive increase in hypothalamic blood-flow.

Discussion. Constriction of hypothalamic vessels as a result of stimulation of the cervical sympathetic is to us the outstanding feature of these experiments. Since the hypothalamic vessels respond with constriction to sympathetic nerve stimulation while medullary vessels do not, it follows that generalizations with respect to the vasomotor innervation of cerebral vessels as a whole cannot safely be drawn from results obtained in one locality only. Investigation of each area separately appears to be necessary. We have already made several accidental observations in experiments in which the instrument was inserted by mistake either anterior or posterior to the hypothalamic area. In two experiments in which it was anterior (temporo-sphenoidal region) sympathetic stimulation caused

vasoconstriction comparable in latency, slow progression, and irreversibility with that observed in the hypothalamus. In three experiments in which the instrument was posterior (upper pons) there was no trace of vasoconstriction from sympathetic stimulation. These highly preliminary results suggest that there may be a fairly sharp demarcation in the distribution of vasoconstrictor fibers from the cervical sympathetic to cerebral vessels.

The physiological significance of this vasoconstrictor innervation is at present uncertain. Strong faradic stimulation of the cervical sympathetic is probably an entirely unphysiological mode of excitation, and it is quite possible that the hypothalamic vasoconstriction so produced would not result from any sympathetic excitation that could arise spontaneously. On the other hand, it is equally possible that a more normal stimulus, or one applied to a more direct nerve pathway, would be more effective than any that we employed. Our attempts at elucidating this point through exciting vasomotor mechanisms by asphyxia or by direct faradic stimulation had an entirely negative outcome. If there is a more direct pathway to the hypothalamic vasoconstrictor innervation than the cervical sympathetic, or a more potent stimulus than faradic excitation of that nerve, we were unable to detect it. Compared with other vascular areas receiving constrictor innervation from the cervical sympathetic, the hypothalamic blood-vessels differ in several important respects: first, they do not dilate when the nerves are cut, as the vessels of muscle or tongue do, so that tonic constrictor impulses apparently do not reach them *via* the nerves; second, stronger currents are needed to cause them to constrict, and the resulting constriction is slower in development and usually more persistent than is the case in the extracranial tissues. For these reasons we are inclined to regard the hypothalamic vasoconstriction as an abnormal response to an abnormal stimulus without counterpart under physiological conditions and without significance to the normal regulation of the circulation in this region. Yet the fact that these vessels can be thrown into a state of sustained contraction by a sympathetic excitation, however abnormal, suggests that a corresponding state might result under abnormal conditions. It is interesting to note that this contraction could be terminated by asphyxia, CO_2, histamine, and at least one derivative of choline, but apparently not by morphine or sodium bromide. It should also be terminated by ergotoxine (ergotamine), but we have been unable to decide whether it actually is or not because blood-pressure always rose markedly after the drug was given and although hypothalamic blood-flow increased, the effect might have been entirely passive. The dosage of ergotamine was enormously greater than any that could be employed for practical purposes, but this is not true of CO_2 or the choline derivative.

The possibility of a humoral intermediary in the vasoconstrictor response

to cervical sympathetic stimulation has already been mentioned (p. 146). It was first suggested by the slow onset, progression, and irreversibility of the response in the hypothalamus. It was strengthened by the rather weak but prolonged vasoconstriction produced by adrenalin (figs. 4, 7, and 9). Additional support was obtained by observations made in the temporal muscle and tongue (fig. 2), in which there was frequently a diphasic effect from cervical sympathetic stimulation—an immediate vasoconstriction followed by partial recovery, then a second wave of vasoconstriction with poor recovery or none at all. It is possible that in the hypothalamus the vasoconstrictor effect of cervical sympathetic stimulation is entirely due to local formation or liberation of a vasoconstrictor substance, while in the extracranial tissues there is an immediate vasoconstriction of direct nervous origin, with a subsequent humoral effect similar to that in the hypothalamus. Of the agents tested, only adrenalin, or a substance acting like it, could be implicated, and one must assume greater intensity and persistence of action than adrenalin displayed when it was injected intravascularly. We prefer to leave this subject open at present, pending the accumulation of more information. It is noteworthy that pituitrin, intraventricular injection of which produces effects which Cushing (1932) attributes to stimulation of parasympathetic centers in the hypothalamus, and calcium, are without constrictor action on hypothalamic blood vessels. The effects of these agents are therefore not due to anemia of hypothalamic centers.

With respect to an intrinsic vascular regulation by CO_2, the situation in the hypothalamus seems to be the same as that in the medulla, and the statements made in the latter connection (Schmidt and Pierson, 1934) are equally applicable here. There is the reservation in the case of the hypothalamus that the dilator effect of CO_2 did not progress as far as it did in the medulla, but this purely quantitative difference does not seem to have great significance. The threshold of sensitivity of hypothalamic vessels to increase in CO_2 content of the blood was identical with that of medullary vessels, i.e., about 1 vol. per cent. The response to oxygen-lack, again at the same threshold as that of medullary vessels (about 12 vols. per cent), seems to be a toxic reaction here as in the medulla. Either CO_2 excess or oxygen-lack in the hypothalamus could lead to increased diversion of blood to it, with CO_2 seeming to be the more important possibility.

We have not yet succeeded in demonstrating a vasodilator innervation to any cerebral vessels, with the possible exception of occasional increases in hypothalamic blood-flow on stimulation of a cervical sympathetic nerve. We do not attach any significance to these because constriction of extracranial areas by the stimulation may well have led to the diversion of blood from them to the intracranial circulation through the abundant anastomoses that exist in the cat. We are convinced that the vago-

depressor and carotid sinus nerves do not carry to hypothalamic vessels vasodilator impulses that can be set up by faradic stimulation, at least in the cat narcotized with barbituric acid derivatives.

We expect to extend these studies to other parts of the brain. Detailed discussion of the literature and of the significance of the results will be postponed until other representative areas have been investigated.

SUMMARY AND CONCLUSIONS

1. A study of the regulation of the circulation in the hypothalamic region has been made in curarized cats narcotized with amytal or pentobarbital, by means of a small artificially cooled thermocouple introduced directly into the tissue.

2. No evidence was found of a vasodilator innervation. Vasoconstriction was quite regularly produced by stimulation of the cervical sympathetic; the effect was slow in onset, it progressed after the stimulation, and it usually was irreversible and not repeatable. Corresponding observations made in the temporal muscle and tongue indicate that these are characteristics of the hypothalamic vessels and not of the recording instrument or nerve excitation employed. A humoral mechanism is suggested.

3. CO_2 excess, oxygen-lack, and asphyxia dilated hypothalamic vessels, CO_2 decrease (increased ventilation) constricted them. Oxygen occasionally constricted them slightly, but changes in pH produced by fixed acid or alkali had no direct effect. CO_2 appeared to have a specific dilator influence here as well as in the medulla.

4. Adrenalin frequently caused relatively weak but rather prolonged constriction of hypothalamic vessels. Pituitrin and calcium had no direct effect. Histamine and a choline derivative were active dilators. The effects of adrenalin, histamine, and the choline derivative were definitely less marked in the hypothalamus than in the temporal muscle or tongue, so that a selective action by any of these agents on the hypothalamic circulation is improbable.

5. At present it appears that the vasoconstriction produced in the hypothalamus by cervical sympathetic stimulation is without great physiological significance in that it can be elicited only by a highly artificial stimulus. Attempts at detecting an intrinsic vasoconstrictor mechanism were unsuccessful. An intrinsic chemical regulation, with CO_2 playing the dominant part, seems more important than nerve control.

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A STUDY OF SUBCUTANEOUS ABSORPTION IN THE ADRENALECTOMIZED RAT[1]

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There are several different points of view as to the nature of the disturbance in adrenal cortex insufficiency. Hartman (1933) subordinates any one site of action of the cortical hormone to a general function in all tissues. The hormone undoubtedly does function in all tissues, but this conclusion does not cast any light on its modus operandi. Rogoff and Stewart (1928) have long favored a toxication theory. Britton and Silvette (1934) emphasize a disturbance in carbohydrate metabolism, supporting their thesis by low values for liver and muscle glycogen, and low blood sugar levels. Other workers have reported occasional low carbohydrate levels, but have not obtained a uniform result, even with animals in extremis. Swingle and co-workers (1934a) have obtained considerable evidence to show that, in the absence of the cortical hormone, there is a profound disturbance in water balance involving the tissues and the blood. Gilman (1934) has produced in normal dogs a condition simulating acute adrenal insufficiency by the withdrawal of large amounts of sodium from the serum. His data indicate that the principal disturbance in this condition is a loss of osmotic equilibrium due to the sodium lack, the normal equilibrium being restored by intravenous injection of hypertonic saline. This finding agrees well with the results of Loeb et al. (1933) and of Harrop et al. (1933): that the kidney excretes an abnormally large amount of sodium in cortical deficiency. It is, however, difficult to reconcile Gilman's conclusions with the recent experiments of Swingle and co-workers (1934b), in which he has shown that cortical extract and water without electrolytes are adequate to restore an animal in advanced cortical insufficiency.

Thus, a review indicates possible disturbances both in water and in carbohydrate metabolism. It was, therefore, thought of importance to study subcutaneous absorption of glucose solutions in adrenalectomized rats, since absorption of glucose involves both the carbohydrate and water exchanges of the body.

METHOD. Rats were adrenalectomized in groups of two or more, and

[1] This study was aided in part by a grant from the National Research Council.

a random number from each group allowed to develop fatal insufficiency. The average life of those rats which survived the operation was seven days. All rats used for the study were autopsied, and careful examination made for regenerated or accessory cortical bodies. It doubtless is true that the rat has many minute secondary cortical bodies, but it is evident from the constant deaths with typical cortical insufficiency that these accessory bodies are not adequate in this emergency.

The method for the determination of subcutaneous absorption was essentially that described by Himwich, Goldman and Krosnick (1932). One cubic centimeter either of a 50 per cent or of a 5 per cent glucose solution in 0.9 per cent saline was injected, the Hagedorn-Jensen (1923) method being used to determine glucose remaining unabsorbed at the site of in-

TABLE 1

Absorption of 50 per cent glucose solution in three hours

CONTROL RATS				ADRENALECTOMIZED RATS				
Rat number	Weight	Blood sugar	Per cent absorbed	Rat number	Days adrenalectomized	Weight	Blood sugar	Per cent absorbed
	grams	*mgm. per cent*				*grams*	*mgm. per cent*	
1	200	112	96	26	4	250	109	82
2	200	158	96	27	4	210	160	82
3	200	110	83	28	4	220	240	76
4	150	125	74	29*	6	300	97	59
5	160		74	30	6	160	92	70
6	300	159	90	31	6	190	138	87
7	260	135	81	32	7	200	140	90
Average.....			85					81

* In extremis; not included in average.

jection. With the extract from the 5 per cent glucose injection, a Somogyi (1930) zinc precipitation was used preliminary to the analysis. This was not done with the 50 per cent solution, but a test made on three filtrates gave identical values both with and without preliminary precipitation. Specific gravities were determined by the method of Barbour and Hamilton (1926).

RESULTS AND DISCUSSION. The results obtained with the 50 per cent solution are listed in table 1. It is quite evident from the data that rats, adrenalectomized from four to seven days, but not in extremis, can absorb in three hours an amount of 50 per cent glucose solution not significantly different from that absorbed by the normals. Rat 29 was prostrate when injected, but seemed much improved after the injection. It will be noted that her absorption was the lowest of the group.

Table 2 shows the absorption percentages obtained with the 5 per cent solution, and again it is apparent that the adrenalectomized rats appearing to be in good condition absorbed the same amounts as did the controls. The three animals, 38, 40, 41, were all in extremis, and it is interesting to note that, although their absorptions were significantly less than the others, they nevertheless averaged the high value of 62 per cent.

The four rats in extremis marked with asterisks in tables 1 and 2 gave somewhat lower values than the others, suggesting that the last stages of the insufficiency were the only ones in which absorption was impaired. To test this, attempts were made to produce shock by withdrawing blood just prior to the subcutaneous injection. For this purpose, the animals were placed under amytal four days after adrenalectomy, while still in

TABLE 2

Absorption of 5 per cent glucose solution in one hour

CONTROL RATS				ADRENALECTOMIZED RATS				
Rat number	Weight	Blood sugar	Per cent absorbed	Rat number	Days adrenalectomized	Weight	Blood sugar	Per cent absorbed
	grams	*mgm. per cent*				*grams*	*mgm. per cent*	
8	260	120	82	33	3	230	132	79
9	250	114	84	34	3	385	142	88
10	220	124	85	35	3	220	157	75
11	290	101	79	36	3	185	129	74
12	355	89	85	37	5	230	110	77
13	310	90	77	38*	5	190	15	59
14	380	127	73	39	7	270	132	76
15	390	111	86	40*	7	225	137	64
				41*	7	225	93	62
Average.....			81					78

* In extremis; not included in average.

good general condition. Laparotomy was performed and varying amounts of blood drawn from the inferior vena cava; the animals were then sewed up and the subcutaneous injection of glucose made. Two minutes before the hour, they were again opened, and the second sample of blood drawn on the hour. For comparison, other rats were subjected to the same procedure with the first bleeding omitted. In this manner, the effects of amytal and laparotomy without hemorrhage were studied.

In all, four groups of rats were treated as described. Of these, the fourth group is presented in table 3 as representative of the results obtained, and shows conclusively that there is no difference in amounts of subcutaneous absorption between adrenalectomized rats, whether bled or not, and their respective controls.

An examination of changes in specific gravities reveals that the adrenalectomized animals reacted differently than did the normals to the same

TABLE 3

Absorption of 5 per cent glucose solution in one hour by rats under amytal

RAT NUMBER	WEIGHT	CONDITION	BLED	SPECIFIC GRAVITY 1	SPECIFIC GRAVITY 2	PER CENT ABSORBED
	grams		*cc. per kgm.*			
16	176	Control	0		1.0233	62
17	195	Control	0		1.0243	68
18	186	Control	0		1.0220	57
Average					1.0232	62
42	216	Adrenalectomized	0		1.0241	67
43	234	Adrenalectomized	0		1.0231	53
44	185	Adrenalectomized	0		1.0214	64
Average					1.0229	61
19	362	Control	8.3	1.0231	1.0209	47
20	324	Control	9.3	1.0238	1.0221	53
21	426	Control	8.0	1.0231	1.0209	49
Average			8.5	1.0233	1.0213	50
45	208	Adrenalectomized	7.2	1.0209	1.0229	57
46	230	Adrenalectomized	8.7	1.0219	1.0289	38
47	206	Adrenalectomized	8.3	1.0212	1.0223	44
Average			8.1	1.0213	1.0247	46

TABLE 4

Recovery from hemorrhage

NORMAL RATS			ADRENALECTOMIZED RATS		
Rat Number	Bled	Remarks	Rat Number	Bled	Remarks
	cc. per kgm.			*cc. per kgm.*	
22	10.4	Respiration good	48	9.2	Shallow, infrequent respiration
23	12.0	Respiration good			
24	14.5	Barely breathing	49	9.3	Died instantly
25	15.2	Died 45 minutes later	50	10.3	Shallow, infrequent respiration
			51	11.5	Died 5 minutes later

proportional amounts of bleeding. Table 3 shows a concentration of the blood in the adrenalectomized rats, as contrasted with a dilution of the

blood in the unoperated rats. This indication that the adrenalectomized animals have lost their capacity to dilute the blood is further supported by the fact, shown by the data in table 4, that they are unable to survive even smaller amounts of bleeding than the normal animals. It is also possible that differences of blood pressure existed, since the adrenalectomized animals never had hemorrhage after the first withdrawal of blood, while it was practically impossible to prevent some additional bleeding in the controls.

Subcutaneous absorption in these rats would seem to depend upon three principal factors: first, a capacity to dilute the hypertonic solutions injected; second, the passage of the diluted material into the subcutaneous capillaries; and third, a circulation adequate to remove material from the area of absorption. If this conception is true, it is surprising to find normal absorption in animals commonly supposed to have a disturbance in these factors.

Analysis of the actual disturbances found here shows that the adrenalectomized rats have lost the power to dilute their blood: the loss of this function would seem to indicate an inability to dilute the hypertonic fluid injected subcutaneously, although some dilution of the glucose solution may take place because of a hydrated condition of the subcutaneous tissues. Since the adrenalectomized animals succumb much more easily to hemorrhage than do the normals, the diminution in blood volume makes for an inadequate circulation. Nevertheless, in spite of the diminished capacity for dilution and the impaired circulation, absorption—the resultant of all three factors of dilution, circulation, and permeability—appears normal. Since this is true, one is forced to admit either that the disturbances found are not sufficiently profound to change the summation effect of all three factors, or that permeability is markedly increased in order to permit adequate absorption.

The first conclusion would depend upon a margin of safety not impossibly large, while the second conclusion involves contradictions. Diminution of blood volume in the adrenalectomized animals may indicate a generalized increase in capillary permeability. Recently, however, the results of Loeb et al. (1933) and of Harrop et al. (1933) indicate a specific kidney change. The alteration of renal function seems to be selective for sodium, as changes for glucose have not been reported.

In view of other investigators' results, the conclusion to be drawn from our finding of practically normal subcutaneous absorption in adrenalectomized rats is that the cortical hormone does not directly regulate capillary permeability. It is evident, moreover, that any derangement of carbohydrate metabolism cannot be attributed to inability of the capillaries to transfer glucose.

CONCLUSIONS

1. Adrenalectomized rats appearing in good condition are able to absorb 50 per cent and 5 per cent glucose solutions injected subcutaneously as well as normals. Even in extremis adrenalectomized rats absorb only slightly less than do controls.

2. Absorption of a 5 per cent glucose solution injected subcutaneously is the same in adrenalectomized rats under amytal as in unoperated rats under amytal.

3. Hemorrhage causes a decrease in subcutaneous absorption by adrenalectomized rats. This decrease is not significantly different from that brought about in unoperated rats.

4. Any derangement of carbohydrate metabolism in adrenalectomized rats cannot be attributed to inability of the capillaries to transfer glucose.

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REACTION OF THE ANTERIOR PITUITARIES OF IMMATURE FEMALE RATS TO INJECTION OF PREGNANCY URINE EXTRACTS[1]

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It has been reported by Wolfe, Phelps and Cleveland (1933) that injections of an extract of human placenta, rich in the A. P. L. factor, induced a marked weight increase of the ovaries and, to a lesser extent, of the pituitaries of immature female rats. Histologically, the anterior pituitaries of these rats exhibited marked granular depletion of the basophiles and to a lesser degree of the eosinophiles. Of equal interest was the fact that, if the animals were castrated at the beginning of the injection, these changes were not induced.

Subsequent studies of Severinghaus (1934) and Wolfe, Ellison and Rosenfeld (1934a, b, c) have demonstrated that injections of a pregnancy urine extract into mature female rats brought about marked changes in the anterior pituitaries. However, such extracts were without action on the anterior pituitaries of female castrates.

In this paper we wish to present results of experiments in which normal immature rats received injections of an extract of pregnancy urine. Thirty-one experimental rats and 33 littermate controls were used. The experimental animals received from 25 to 75 units of an extract[2] of pregnancy urine daily for 7 to 15 days. Injections were begun on the 21st to the 25th days of life.

At autopsy the ovaries and the pituitaries were weighed on a torsion balance (Hartmann-Braun, accurate to 0.5 mgm.). The ovaries of the experimental animals were markedly increased in size, containing many large corpora lutea and follicles. The weight of the ovaries ranged from 50 to 102.5 mgm.; the mean was 71.4 mgm., with a standard deviation (S. D.) of 4.5 (see table 2). These animals received from 25 to 75 units of the

[1] These studies have been aided by grants from the Committee for Research in Problems of Sex of the National Research Council, the Committee for Scientific Research of the American Medical Association, and from the Division of Medical Sciences of the Rockefeller Foundation.

[2] Pregnancy urine extract, Follutein, was furnished by E. R. Squibb and Sons through the courtesy of Dr. J. J. Durrett.

extract daily; within these limits the amount of extract injected daily had no definite effect on the final weight of the ovaries; i. e., 25 units were as effective as 75 units. The weight of the ovaries of the controls ranged from 14 to 25 mgm.; the mean was 19.3 mgm., with a standard deviation of 3.0 (table 2). In table 1 detailed data on the weights of the ovaries and the pituitaries of a representative group of experimental rats and their controls are given.

The pituitaries of the injected rats were also definitely heavier than those of the controls. The mean weight of the pituitaries of the injected rats was 3.9 mgm. (standard deviation of 0.5); that of the controls was 2.7 mgm. (standard deviation of 0.9). Furthermore, this pituitary weight

TABLE 1

Detailed quantitative data on representative injected and littermate control rats taken from two litters

TYPE	INJECTION PERIOD	DOSAGE R. U.	ANIMAL WEIGHT	OVARY WEIGHT	PITUITARY WEIGHT	PERCENTAGE OF CELL TYPES				CELLS COUNTED
						Eosin.	Basophiles		Chrom.	
							Gran.	Non-gran.		
			grams	*mgm.*	*mgm.*					
Control			64	19	3.5	33.0	4.3	1.7	60.8	1,358
Control			64	15	3.5	34.5	6.7	2.0	56.8	1,799
Experimental	7	50	70	86	4.0	22.0	2.9	5.3	69.8	1,881
Experimental	7	50	72	100	4.5	24.0	2.6	4.3	68.0	2,078
Control			58	22	2.0	38.8	9.1		52.1	1,401
Control			64	18	2.0	37.6	8.8	1.2	52.4	1,472
Experimental	7	75	60	65	3.0	29.0	2.3	4.3	65.3	2,653
Experimental	7	75	60	80	3.0	27.0	2.7	4.9	65.4	2,461

increase was greatest in experimental rats presenting the heaviest ovaries. This confirms findings in immature female rats which received placental extracts, rich in the A. P. L. factor (Wolfe, Phelps and Cleveland, 1933) and in mature female rats which received injections of pregnancy urine extracts (Wolfe, Ellison and Rosenfeld, 1934b, c).

At autopsy, the ovaries and accessory reproductive organs were fixed in Bouin's fluid, and stained in hematoxylin and eosin. The pituitaries were fixed in Regaud's fluid and prepared for study by methods already described (Cleveland and Wolfe, 1932). Cell counts were made on all sections studied; a total of 140,009 cells was counted in this series.

In the anterior pituitaries of the 33 immature control rats, the percentages of the eosinophiles ranged from 30.1 to 44.9; the mean percentage was

36.1, with a standard deviation of 3.9 (see table 2 for frequency distribution and statistical data). These cells were usually well packed with

TABLE 2

A statistical analysis of the quantitative data on immature control and injected rats

PERCENTAGE OF CELLS	FREQUENCY		MEAN—STANDARD DEVIATION (PER CENT)	
	Controls	Experimental	Controls	Experimental
Eosinophiles:				
20.0–24.9		7	Mean 36.1	Mean 27.2
25.0–29.9		20	S.D. 3.9	S.D. 3.1
30.0–34.9	16	5		
35.0–39.9	10			
40.0–44.9	7			
Basophiles granular:				
0.0– 0.9		5	Mean 7.2	Mean 2.2
1.0– 1.9		6	S.D. 1.3	S.D. 0.9
2.0– 2.9		15		
3.0– 3.9		6		
4.0– 4.9	2			
5.0– 5.9	8			
6.0– 6.9	8			
7.0– 7.9	7			
8.0– 8.9	5			
10.0–10.9	1			
Basophiles non-granular:				
0.0– 0.9	10	2	Mean 1.9	Mean 4.0
1.0– 1.9	8	3	S.D. 1.2	S.D. 1.6
2.0– 2.9	10	2		
3.0– 3.9	2	6		
4.0– 4.9	3	11		
5.0– 5.9		5		
6.0– 6.9		3		
Chromophobes:				
45.0–49.9	4		Mean 55.0	Mean 66.3
50.0–54.9	11		S.D. 3.2	S.D. 3.1
55.0–59.9	15	1		
60.0–64.9	3	7		
65.0–69.9		20		
70.0–74.9		3		

granules which stained intensely with orange G. The level of the basophiles ranged from 6.0 to 10.8 per cent. A great majority of these were well packed with granules. The mean percentage of the cells filled with

granules was 7.2 (standard deviation of 1.3), while the mean level of those exhibiting granular loss was 1.9 per cent, with a standard deviation of 1.2. See table 2 for frequency distribution and statistical data.

Histologically, the anterior pituitaries of the injected rats were markedly different from those of the control rats. Most outstanding were changes in the basophiles, which were considerably enlarged and exhibited evidence of granular loss. Considering the experimental group of rats as a whole, the mean percentage of basophiles well filled with granules was only 2.2, while the mean of those in which there was loss of granules was 4 per cent. This loss of granules from the basophiles in the experimental rats was associated with a decrease in the total percentage of the basophiles. In two or three instances control rats were encountered which exhibited granular depletion in a majority of the basophiles. See table 2 for statistical data on the basophiles in the experimental and control groups. In table 1, detailed quantitative data are given on two representative litters of individual experimental and control rats.

The anterior pituitaries of the injected animals also presented marked but less striking changes in the eosinophiles. Many of these cells were swollen and presented evidence of granular depletion, which in some cells was moderate and in others extreme. In the cells exhibiting granular loss, the remaining granules were less closely spaced and took a pale stain with orange G. In many cells this loss of granules was extreme. In many of the eosinophiles showing granular depletion, the negative image of the Golgi apparatus, which usually appeared as a semilunar system of clear canals capping the nucleus, was enlarged. Associated with the loss of granular material from the eosinophiles was a decrease in the relative percentage of the eosinophiles and a corresponding increase in that of the chromophobes. Considering the 31 experimental rats as a group, the mean level of the eosinophiles was 27.2 per cent, with a standard deviation of 3.1 per cent; in the controls the mean level of these cells was 36.1 per cent. Full statistical data are given in table 2 and detailed data on two representative litters of rats are given in table 1.

Associated with the decrease in the percentages of the basophiles and eosinophiles in the experimental rats, there was an increase in the chromophobes. In the control rats the mean level of the chromophobes was 55 per cent, while in the experimental rats the mean level of these cells was 66.3 per cent (see table 2). The chromophobes appeared in some instances as large clear cells with a grayish-blue cytoplasm which was usually fragmentary. Other chromophobes were small, with a rather dense blue cytoplasm. In still others, the cell membrane was indefinite and the nucleus was surrounded by a few pale strands of cytoplasm. In many of these the negative image of the Golgi apparatus was observed.

Mitoses were abundant in the anterior pituitaries of both the controls

and experimental rats, although they were somewhat more numerous in the pituitaries of the injected rats. In the sections (on which cell counts were made) of the injected rats, 444 mitotic figures were counted, while in those of the controls 264 were counted. A majority of the mitoses was in chromophobes, a lesser number was in the eosinophiles; mitoses were not observed in basophiles.

DISCUSSION. These experiments are similar to those recently described (Wolfe, Ellison and Rosenfeld, 1934b, c) in which changes, resulting in the anterior pituitaries of mature female rats from injections of pregnancy urine extracts,[3] were pointed out. Qualitatively, the results were identical; there was loss of granules from both the basophiles and the eosinophiles in both groups. However, the quantitative changes were more marked in the anterior pituitaries of the mature female; i.e., the levels of the basophiles and the eosinophiles were more markedly reduced and the level of the chromophobes more markedly increased. Analysis of table 2 presented in this paper and those in the papers mentioned above will reveal the fact that the results obtained by injecting pregnancy urine were much more constant in immature rats than they were in mature rats. In the mature rats, the weight of the ovaries of the injected animals varied from 106 to 766 mgm., and the pituitaries from 8 to 26 mgm., while in the immature animals the ovaries varied from 50 to 100 mgm. and the pituitaries from 2.5 to 5.0 mgm. Histological comparison of the two groups of anterior pituitaries revealed that the granular depletion was much more marked in the anterior pituitaries of injected mature rats than in immature rats. However, the degree of depletion in these cells was more uniform in the immature rats. Although the reason for the more uniform results obtained in the immature rats is certainly not known, it would seem that the lack of a reproductive cycle prior to the injections would play some rôle.

These experiments are of considerable interest in that the results obtained are similar to those obtained by injecting extracts of human placenta, rich in the A. P. L. factor, into immature female rats. In both groups, the ovaries were markedly enlarged, presenting many large corpora lutea and follicles. In both groups the pituitaries were moderately enlarged and, histologically, exhibited loss of granules from both the eosinophiles and the basophiles; this loss was most striking in the case of the basophiles. Such findings would give some indication that the factors responsible for these changes were the same in both instances.

[3] We pointed out in this paper (Wolfe, Ellison and Rosenfeld) that the anterior pituitaries of mature female rats receiving such injections were similar to those of rats killed in early pregnancy and pseudopregnancy (first 6 days). However, specialized "pregnancy cells" have never been observed. We have discussed the so-called pregnancy cell fully in a previous paper (Wolfe and Cleveland, 1934, *Anat. Record* **56**: 33–45).

SUMMARY AND CONCLUSIONS

Thirty-one immature female rats (21 to 25 days) received from 25 to 75 units of pregnancy urine extract daily for 7 to 15 days. Thirty-three littermate sisters served as controls. The ovaries of the injected animals were markedly increased in weight, while the pituitaries were moderately increased. Histologically, the anterior pituitaries of the injected rats exhibited a loss of granules from the eosinophiles, which were also decreased in relative percentage. Most striking changes were found in the basophiles, which were enlarged and exhibited *marked* loss of granules. They were reduced in percentage. The percentage of the chromophobes was increased. Mitoses were more abundant in the anterior pituitaries of the injected rats. Cell counts were made on all sections studied; a total of 140,009 cells was counted.

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VERTEBRATE NERVES: SOME CORRELATIONS BETWEEN FIBER SIZE AND ACTION POTENTIALS

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Certain proposals were made by Gasser and Erlanger (1927) to describe the relationships existing between the diameters of myelinated fibers and their potentials following stimulation. This analysis was not carried with precision below 5μ axons for most of their nerves, since the potentials then to be accounted for did not demand it, and in particular, non-myelinated fibers were not counted. Since then, further information concerning the more slowly conducting processes of autonomic nerves calls for a more detailed analysis of the small fiber range. Two difficulties that arise, the accurate sorting into size groups of the smaller fibers, and the monophasic recording of their action potentials, have not been completely overcome. Since conduction time, which is the factor separating out the potentials in the nerve record, tends to vary inversely as the diameter, an accurate matching of the potential against fiber groups requires measurement by smaller intervals in the small fiber range, where the percentage error of measurement of fiber size is greatest. On the other hand, it has been impossible so far to obtain strictly monophasic records of the slower potential waves for plotting against fiber distribution, and due to the presence of relatively large after-potentials, both positive and negative, it is not certain where the main potential ends and the after-potential begins. The following data are therefore presented as only qualitatively significant.

Gasser and Erlanger gave as the nearest simple relations between size of axon and potential of the larger fibers, that the amplitude of potential varied as the cross-sectional area of the fiber, that is, as the diameter

[1] The histological technic and counting and classifying of nerve fibers was done by Douglass (as part of the requirements for the degree, Master of Science) and Davenport at Northwestern University on nerves supplied by Heinbecker and Bishop, who obtained the oscillographic records at Washington University. The two phases of the work were thus entirely independent until the final stage of combining the data was reached.

[2] Contribution no. 209.

squared, and conduction rate varied as the diameter, measurements being made of the outside of the myelin sheath. More recently Blair and Erlanger (1933) have suggested that conduction rate also varied more nearly as the square of the diameter than the diameter. We plot the data that follow on the basis of the linear diameter, leaving the graphs (figs. 1 to 8) to indicate the deviation from this rule in the small fiber range. For the use we wish to make of this material the exact conduction rate relationship is not important, but only that the separation of the groupings of the fibers by size should clearly correspond to the separation out of the potential waves by differences of rate.

When Gasser and Erlanger proposed that amplitude of potential varied as cross sectional area of the fiber, it was believed that the durations of all potentials were the same, and this is approximately true for the potentials of the larger fibers they dealt with. The longer durations of the potentials of the axons of the autonomic system (Bishop and Heinbecker, 1930) and of the dorsal root C fibers, occasion some doubt as to the propriety of extending their inferences to fibers other than those on which they were based. For technical reasons, moreover, it is simpler to obtain the total area of a potential than the amplitude of the axon potential, and since figures for the durations are available (Bishop and Heinbecker, l.c.) and confirmed, with reservations, by Blair and Erlanger (1933) the amplitude may be estimated for the axon action potential. In the following we propose to compare the *area* of potential per fiber for fibers of different size, and have done this by comparing the areas of the different potential waves with the sums of the areas of the fibers to which these waves appear to correspond.

An error is introduced by comparing the tracing of the potential picture as recorded (the single-fiber potentials of which have different durations of from 1 to perhaps 10 sigmas) with the *fiber-area* curve plotted as a series of points. Ignoring the duration of the individual fiber potentials however, the fault lies chiefly in comparing the total area of potential instead of the amplitude of potential, with fiber area. The slight lateral relative displacement of the curves is of minor importance for the purposes involved, since it does not obscure the correspondence between fiber size areas and potential areas.

Technic. Action potential records were obtained with a cathode ray oscillograph from the following nerves: saphenous, phrenic and sympathetic of cat; phrenic, sympathetic and depressor of rabbit; and two vagi of turtle. The nerves were rendered as monophasic as crushing would make them, and since this was not complete, the distance between proximal lead and killed region was made as large as practicable to allow the first phase to be recorded before the second commenced. Since the separation of discrete waves depends upon distance of conduction from stimulus to

proximal lead, a limit was imposed upon the amount to be left between leads. In recording some nerves, the potentials required for comparison were confluent; the first wave was recorded at its maximum, then the first and second together, and the area of the second obtained by subtraction. The nerves were subsequently fixed two to three days in a solution of 10 per cent formalin plus 3 per cent acetic acid, then washed and cut in two. One end was stained with osmic acid and the other embedded unstained in paraffin. Sections 5μ thick from the adjacent ends of both pieces were cut and the unstained sections stained with silver by Davenport's (1930) method. Thus closely adjacent cross sections of the nerves were obtained, one of which was used for counting and measuring the myelinated fibers and the other for counting the total number of axis cylinders. The difference between the two numbers was taken as the number of unmyelinated fibers.

Myelinated fibers were measured on photographs by using circular loops of 26 gauge wire. The photographic enlargement was between 1000 and 2000 and the loops gauged to even millimeters. The measurements were reduced to fiber diameters in micra by dividing the size measured in millimeters by the magnification of the photograph. When fibers were not round, the criterion of size was taken as that of the loop which excluded enough of the cross section of the fiber to approximate the area within the loop not occupied by the fiber. This method was compared with measurements made by the method of Gasser and Erlanger (1927) and found to vary plus or minus 0.25 micron in medium size fibers.

Axons were counted by observing the section microscopically under oil-immersion and checking off each fiber on a photograph as counted. Observation under oil was necessary to resolve the unmyelinated fibers, while comparison with the photograph insured accuracy by obviating omissions and the counting of a fiber twice.

Myelinated fibers were classified into groups whose external diameters were within one micron of each other, except those below three micra. These were measured with a filar micrometer and classified into groups having either 0.5 or 0.8 micron intervals.

Tracings of action potential curves have been plotted to a scale which can be compared readily with the graphs of fiber size distribution. Rectangular linear coördinates were used. The solid line indicates the record of action potential and the dotted line the distribution of myelinated fibers. Ordinates are used for millivolts and for the diameter of fiber squared times the number (d^2n). Abscissae indicate conduction time in sigmas and diameter of fibers. No attempt was made to measure the size of unmyelinated fibers, hence these were plotted as a rectangular graphical representation of their number to permit easy comparison with the corresponding C wave.

On the graphs, the minima between potential waves and fiber distribution groups were taken to correspond, and the areas of the fibers were compared with the potential areas in the following manner, taking for illustration the data pertaining to figure 1, of the cat saphenous. d^2n was computed for the fibers of the A and B groups. The potential areas were measured by planimeter in arbitrary units. These values were expressed in per cent of the total area assignable to myelinated fibers. The percentage *fiber* area of A divided by that of B = the percentage potential area of A divided by that of B times a constant K, in this case K = 1.5.

TABLE 1

Comparisons of potential areas with fiber areas

NERVE	PERCENTAGE AREA						K	PERCENTAGE C POTENTIALS OF TOTAL	C VALUES		
	Fibers			Potentials					Equiv. d^2, $\mu 2$	Equiv. d, μ	Equiv. axon d, μ
	A	B_1	B_2	A	B_1	B_2					
1. Saphenous of cat	91	9		87	13		1.5	20	5.10	2.26	1.6
4. Sympathetic and depressor of rabbit		53	47		27	74	3.1	*			
5. Depressor only of 4		80	20		93	7	0.3†	40	3.15	1.78	1.26
5a. Sympathetic only‡			100					49	0.55	0.74	0.53
7. Vagus of turtle, 1.		58§	42				1 to				
		70	30		55	45	1.6	90	47.0	6.8	4.85
8. Vagus of turtle, 2.		50	50		70	30	0.4				
		66	34		58	42	1.4	70	25.2	5.0	3.55

* C record complicated by after-potential, equivalent axon diameter between 0.7 and 1.8 μ.

† B_2 diphasic and small, measurement approximate.

‡ Record is not given in the figures.

§ Two parts of B wave confluent, two possible extremes measured, division of fiber groups questionable, K value lies between those given.

This means that the potential area per unit fiber area for the B group is 1.5 times that for the A (nerve 1, table 1).

Since the *C* fibers in silver preparations are shrunken more than the others in osmic, this procedure cannot be applied to them. However, if the same computation is made backwards, having given the area of potential and the number of fibers, and assuming the same potential per unit cross-section for myelinated and unmyelinated fibers, one may derive the value of d^2. This proves to be in most cases greater than 1 mu, to give the action potential area obtained, if the potential area per unit cross-section were the same as for myelinated fibers (C values, table 1). For instance,

in figure 1, the area of the A plus B potentials was 80 per cent, that of the C, 20 per cent of the total. The non-myelinated fiber area computed from this, divided by the number of non-myelinated fibers, is 5.10, which is the hypothetical average of d^2. The square root of this, 2.25 μ, may be called the *equivalent* non-myelinated fiber diameter. Since however the myelinated fibers are about one-half myelin, the equivalent *axon* diameter should be the square root of one-half the above square, or 1.6 μ. That is,

TABLE 2

Conduction rates of waves of action potentials and diameter of fiber at the maxima of fiber size curves corresponding to these waves

NERVE*	A		B_1		B_2		B_3		C
	CONDUCTION RATE	SIZE	CONDUCTION RATE	SIZE	CONDUCTION RATE	SIZE	CONDUCTION RATE	SIZE	CONDUCTION RATE
	M/sec.	μ	*M/sec.*	μ	*M/sec.*	μ	*M/sec.*	μ	*M/sec.*
1. Saphenous of cat..	75–30	11.8	18–11	3.2					0.9–0.7
2. Phrenic of rabbit..	77.8–27.7	10.8							
3. Phrenic of cat.....	87.5–37.2	10.4	24.1–10.3	?					
4. Sympathetic and depressor of rabbit.....			27.5–11.9	2.6	11.9–5.9	1.0			1.7–1.1
5. Depressor of rabbit.			27.5– 7.6	2.6	11.9–5.0	1.5(?)			1.8–1.2(C_1) 0.9–0.7(C_2)
6. Sympathetic of cat.			20.1– 3.3	2.5					1.0–0.8(C_1) 0.7–0.6(C_2)
7. Vagus (1) of turtle..			29– 3.3	4.1			2.4–1.4	1.6	0.6–0.1
8. Vagus (2) of turtle.....			29.5– 5.8	5.8	5.5–2.9	3.3	2.4–1.9	1.7	0.9–0.4

* The numbers of myelinated (all sizes) and unmyelinated fibers for the nerves are as follows: 1. M 2249, U 6790; 2. M 759, U 62; 3. M 755, U 6; 4. M 1391, U 2222; 5. M 380, U 431; 6. M 2005, U 1663; 7. M 5101, U 5279; 8. M 4363, U 4958.

the non-myelinated axons whose axon is 1.6 μ in diameter give a potential area per axon equal to a myelinated fiber 2.25 μ in diameter. While there is some uncertainty as to how large non-myelinated fibers really are, they certainly do not average this size. It appears therefore that non-myelinated fibers give more potential per cross-sectional area than do myelinated.

Discussion. Two qualifications are necessary in interpreting the data of the tables 1 and 2. First, the validity of the computations depends on the assumption that potential groups correspond to fiber groups, and in

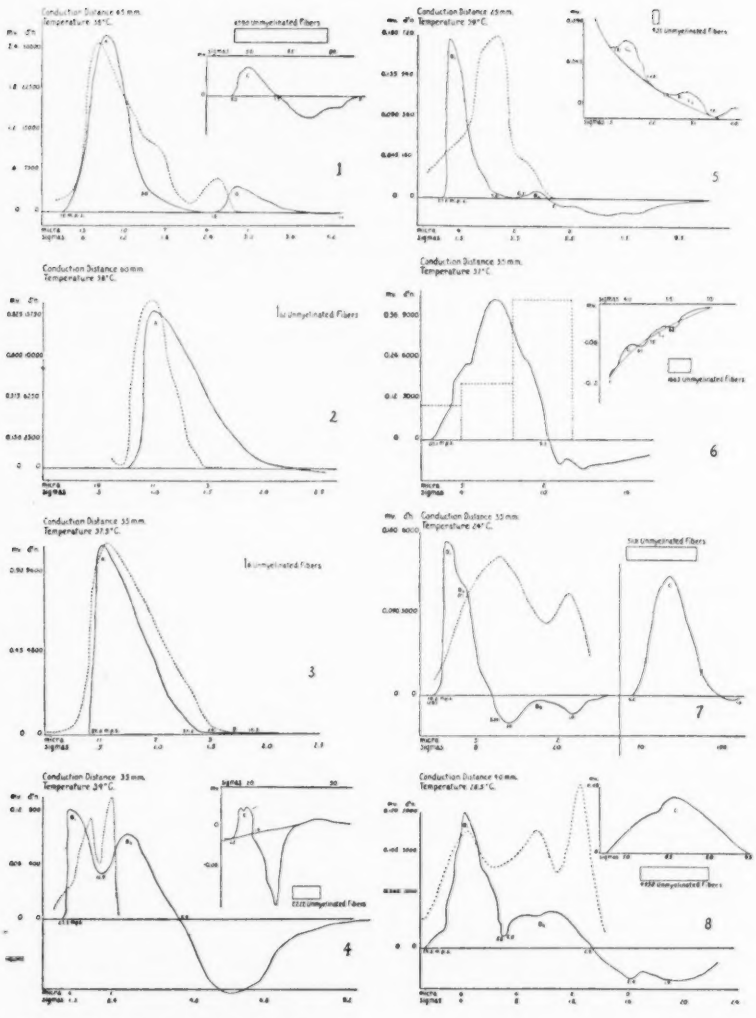

Fig. 1. Saphenous nerve of cat.
Fig. 2. Phrenic nerve of rabbit.
Fig. 3. Phrenic nerve of cat.
Fig. 4. Combined depressor and cervical sympathetic nerve of rabbit.
Fig. 5. Depressor nerve of rabbit.
Fig. 6. A sympathetic nerve of cat.
Fig. 7. Vagus nerve of turtle (1).
Fig. 8. Vagus nerve of turtle (2).

———— Action potential, millivolts and sigmas.

– – – Size myelinated fibers, d^2n = diameter squared times number plotted against diameter.

Numbers along the action potential curves indicate conduction velocity, M/sec.

particular that the C wave corresponds to non-myelinated fibers. The figures must speak for themselves; we wish merely to point out that the C potentials do correspond roughly to the relative numbers of C fibers, and in particular, that for the two phrenics (figs. 2 and 3), from which no C potentials could be recorded, only 6 and 61 non-myelinated fibers were found. These are not enough to give a recognizable potential, for relatively low voltages are recorded from nerves having from 1000 to 5000 such fibers. The fact that the C potential is of long duration, so that its area may be large, does not help in its recognition if the amplitude of its voltage is very low.

Second, not all experiments are equally significant in deriving the values for the potential per fiber area. The ideal condition would be found in a nerve which showed well separated potentials, of about equal areas. This combination is rare. If the potentials are confluent, the separation of both potential areas and fiber areas is uncertain, and if a potential is low, and especially if it is slightly diphasic, a small error in drawing the base line from which to measure its area, may make a large error in its total area, and a still larger error in its relative.

In the values for K, table 1, the ratio of the potential per unit fiber area of the B potentials to that of the A or those of B_2 to B_1 varies from 0.3 to 3.1, but in the nerves where it can be measured with least uncertainty it falls above 1. The "equivalent axon diameter" of the C fibers again falls above 1 μ, in the nerves where the C potential is best developed. Since the C fibers are not 1 μ in average diameter this means the same thing as a K value above 1, that the smaller fiber gives a larger potential per unit cross section. It may be noted that the computations for the C values are based upon the measurements of the total potentials of all myelinated fibers, in which there is less error than in measurements of A or B separately; if the C potentials were compared to the A or B_1 only, the potential per fiber would be larger than the value given; if compared to the B_2 it would be smaller.

The non-myelinated fibers of turtle nerves appear to be larger in cross sections stained with silver than those of mammals. The values of the potential per fiber area are also larger. Both potentials however are too large to correspond to the fiber sizes as compared to the potentials of myelinated fibers in the same nerves. The slower potentials are in general those most easily depressed, so that any error due to differential depression would make the C values smaller, relatively to the A and B. The C potentials also are strongly diphasic, under conditions in which the earlier waves are monophasic or nearly so, and if the second phase cut the first short, this again would *reduce* the C potential per fiber area as measured.

The relation of conduction rate to fiber size is shown in table 2 and the qualitative agreement of our data with previous work, to the effect that

conduction rate varies as a function of size between the linear and the square of the diameter, is indicated. It may be however that the increasing *area* of potential per fiber area, as the *amplitude* of potential *decreases* toward the small fiber range, has some significance here. The simplest conception of the rationale of the relation is perhaps that of Gasser and Erlanger to the effect that, since the surface of a fiber varied as the diameter, but the electrical conductance as the square, if all fibers produced the same intrinsic potential force, the current density across the fiber surface, ahead of an active region, would vary as the first power of the diameter, and this might be the factor controlling the relation of conduction rate to size.

It appears however that the smaller the fiber, the longer the duration of its potential (Bishop and Heinbecker, 1930) and if the intrinsic potential force of all fibers were the same, and the amplitude therefore varied as the diameter squared, a longer duration of potential in smaller fibers would result in a larger area of potential per fiber area. We find to be sure such an increase, but not enough to compensate for the longer duration of potential that occurs. This requires that the amplitude fall off faster than the square of the diameter; whether this means that the intrinsic potential force *decreases* as size decreases, instead of remaining constant as assumed, or whether some other factor is involved, cannot be said. It may mean however that the effective flow of current that is supposed to stimulate the fiber adjacent to its active region decreases more rapidly than the simple theory supposes, that is, more rapidly than a linear function of diameter.

If, for instance, the duration of potential increased inversely as the diameter, while the intrinsic potential force remained constant, conduction rate on the basis of the assumptions made should vary as the square of the diameter instead of the diameter; provided the area of potential per fiber area were constant. Since, however, this area increases as size decreases, conduction rate should be expected to decrease less steeply than the diameter squared. The fact that rate varies by a function between the linear and the square of the diameter is therefore quite consistent, both with the original assumptions as to the physics of nerve, and with the subsequent data obtained as to its potentials.

We wish, however, to imply no final limit to the number of variables that affect nerve functioning, especially since the description of that functioning is not yet complete. We therefore repeat, our results are consistent with certain assumptions, but do not thereby establish their validity.

SUMMARY

1. Unmyelinated fibers were counted and myelinated fibers counted and measured in a saphenous, phrenic and sympathetic nerve of cat; a phrenic, sympathetic and depressor nerve of rabbit; and two vagi of turtle.

2. A classification of fiber sizes was plotted and compared with action potential waves.

3. Although, in general, maxima in the fiber size distribution corresponded with an equal number of the potential maxima and had a comparable order of magnitude, no function of fiber size was found from which potential waves could be predicted accurately.

4. Conduction rate diminished more rapidly in the smaller sizes of fibers than would be expected if the conduction rate bore a linear relationship to the size of fiber.

5. The presence of C waves in the action potential of all nerves investigated, excepting the phrenic nerves, seems to confirm previous conclusions that the C wave is due to unmyelinated fibers.

6. The isolated group of fibers with diameters 2.6 to 5.2 micra in the depressor of the rabbit, together with the appearance of a large B_1 wave seemed to indicate that this wave is due to fibers between these sizes. As a corollary, the large B_2 wave observed in the combined sympathetic and depressor was produced by fibers less than 2.6 micra in diameter.

7. The large amplitude of C waves in turtle vagi was associated with large size unmyelinated fibers.

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THE PROTEIN CONTENT AND OSMOTIC PRESSURE OF BLOOD SERUM AND LYMPH FROM VARIOUS SOURCES IN THE DOG

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The relative proportions of the different protein fractions which go to make up the total protein concentration of lymph and the colloid osmotic pressure of lymph have received very little attention in the literature. This has been due largely to the difficulty of obtaining any but thoracic duct lymph in quantities sufficient for separation and analysis.

Munk and Rosenstein (1891) reported two analyses of lymph obtained from a fistula in the leg of a young girl. The total protein concentration was 3.5 per cent; in one sample the albumin/globulin ratio was 4.0, and in the second, 2.4. Morawitz (1906) in two experiments on dogs, compared the albumin/globulin ratios of blood plasma and thoracic duct lymph. In one the albumin/globulin ratio of blood was 1.25, of lymph, 1.56; in the second the albumin/globulin ratio of blood was 1.44, and of lymph, 1.58.

Loewen, Field and Drinker (1931) measured the colloid osmotic pressure of serum, cervical lymph, and thoracic duct lymph in a series of dogs. They observed that the osmotic pressure of lymph per gram of protein was always considerably higher than that of serum, and suggested that this might be due to a greater permeability of the blood capillary for serum albumin. Their protein determinations were made refractometrically and no measurements were made of the amounts of albumin and globulin present.

The albumin/globulin ratios of serum and lacteal lymph, together with measurements of the colloid osmotic pressure, were determined by Wells (1932) in a series of dogs. The average albumin/globulin ratio of serum was 1.18; and of lacteal lymph, 139. The osmotic pressure per gram of protein was found to be practically the same for serum and lymph.

Weech, Goettsch and Reeves (1933) compared the protein fractions of serum with those of lymph obtained from vessels draining the paw and lower leg of the dog. They found the albumin/globulin ratios of lymph slightly higher than those of the corresponding serum.

In view of the different results for specific osmotic pressure obtained by Loewen, Field and Drinker (1931) and Wells (1932), it seemed worth while

to compare the albumin/globulin ratios and osmotic pressures of serum with those of lymph obtained from as many different sources as possible in the same animal.

METHODS. *Collection of lymph.* Leg lymph was collected from walking dogs, the lymphatics being cannulated under local anesthesia (novocaine) as described by White, Field and Drinker (1933).

At the conclusion of the period of collecting leg lymph, the animals were anesthetized intravenously with nembutal. Cervical lymph was collected from the cannulated cervical vessels by gentle massage. Thoracic duct lymph was obtained from the duct cannulated at its entrance into the junction of the jugular and subclavian veins. After sufficient cervical and thoracic duct lymph had been obtained for analyses, the abdomen was opened and the lymphatics from the liver which run along the portal vein were exposed and cannulated. Through the same exposure it was possible in two cases to obtain intestinal lymph by cannulating a large lymphatic just before its entrance into the thoracic duct. Trypan blue injections in the liver and intestine at the end of the experiment confirmed the drainage areas. The lymph collected was allowed to clot spontaneously and the thin clot removed by centrifuging.

Collection of blood. Samples were taken from the jugular vein. The blood was allowed to clot, centrifuged at high speed for ten minutes and the serum removed at once.

Determination of the total protein and albumin and globulin fractions of lymph and serum. The total protein was calculated as 6.25 times the difference between total and non-protein nitrogen which was determined by micro-Kjeldahl method.

Non-protein nitrogen determinations were made on tungstic acid filtrates, prepared according to the method of Folin and Wu (1919). Determinations could not be carried out on every sample of lymph, but always on serum and at least one lymph sample from each dog. The other lymph samples were arbitrarily corrected.

The globulin fraction was removed with 22.2 per cent sodium sulfate solution, as suggested by Howe (1921), and as modified by Bruchman, D'Esopo and Peters (1930). The nitrogen was determined by a micro-Kjeldahl method, a modified Pregl apparatus being used for distillation. The albumin was calculated as 6.25 times the difference between the total nitrogen remaining after removal of the globulin and non-protein nitrogen. The globulin was calculated by difference. Duplicate determinations were invariably made.

Determination of the colloid osmotic pressure of serum and lymph. These were carried out by the method of Krogh and Nakazawa (1927) as described by Turner (1932). Cellophane no. 600 was used for the membrane. Duplicate determinations were made with one or two exceptions. The

final readings were made after 20 and 22 hours. The greatest variation between the pairs was 16 mm. of water, the least variation zero, and the average 5.0 mm. of water.

TABLE 1

Concentration and osmotic pressure of proteins in serum and lymph. Arranged in order of ascending albumin/globulin ratios

NUMBER OF DOG	BLOOD SERUM						
	Albumin	Globulin	A/G ratio	Total protein	Total osmotic pressure	Osmotic pressure per gram protein	Non-protein nitrogen
	grams per cent	*grams per cent*		*grams per cent*	*mm. H_2O*	*mm. H_2O*	*mgm. per cent*
1	3.19	4.45	0.97	7.64	325	42.5	50.8
2	3.54	3.02	1.17	6.56	343	52.2	35.0
3	3.33	2.79	1.19	6.12	222	36.2	40.0
4	3.70	2.99	1.24	6.69	294	43.9	26.3
5	3.55	2.61	1.36	6.16	339	55.0	37.1
6	3.46	2.45	1.41	5.91	306	51.7	31.6
7	3.33	2.33	1.43	5.66	232	40.9	49.2
8	3.46	2.42	1.43	5.88	369	62.7	43.2
9	3.34	2.28	1.46	5.62	291	51.7	35.3
10	3.78	2.52	1.50	6.30	330	52.3	44.0
11	4.01	2.40	1.67	6.41	302	47.1	36.0
12	3.95	2.11	1.87	6.06	301	49.6	37.7
13	4.36	1.91	2.28	6.27	336	53.5	35.1
Average...	3.61	2.63	1.46	6.25	306	49.1	38.9

NUMBER OF DOG	CERVICAL LYMPH							
	Albumin	Globulin	A/G ratio	Lymph/ serum ratio	Total protein	Total osmotic pressure	Osmotic pressure per gram protein	Non-protein nitrogen
	grams per cent	*grams per cent*			*grams per cent*	*mm. H_2O*	*mm. H_2O*	*mgm. per cent*
1	2.67	2.30	1.16	0.65	4.97	237	47.6	
2	2.00	1.21	1.65	0.49	3.21	203	63.2	30.0
3	2.51	1.51	1.65	0.66	4.02	208	51.7	40.0
4	1.69	0.89	1.90	0.39	2.58	98	37.6	
5	2.38	1.43	1.66	0.62	3.81	168	44.0	
6	1.70	1.07	1.59	0.47	2.77	96	34.6	40.7
7	2.05	1.28	1.60	0.59	3.33	118	35.4	48.0
8	2.49	1.33	1.87	0.65	3.82	189	49.4	
9	2.89	0.85	3.40	0.67	3.74	148	39.5	38.2
10	2.31	1.15	2.00	0.55	3.46	137	39.5	
11	2.62	1.34	1.95	0.62	3.96	153	38.6	34.6
12	1.90	0.88	2.16	0.46	2.78	122	43.8	32.2
13	3.52	1.22	2.89	0.76	4.74	209	44.0	35.1
Average..	2.36	1.26	1.96	0.58	3.63	160	43.7	37.4

TABLE 1—*Continued*

NUMBER OF DOG	LEG LYMPH							
	Albumin	Globulin	A/G ratio	Lymph/ serum ratio	Total protein	Total osmotic pressure	Osmotic pressure per gram protein	Non-protein nitrogen
	grams per cent	*grams per cent*			*grams per cent*	*mm. H_2O*	*mm. H_2O*	*mgm. per cent*
1	1.14	1.12	1.02	0.30	2.26	106	46.9	
2	1.21	0.92	1.32	0.32	2.13	113	53.0	
3	1.63	0.90	1.81	0.41	2.53	110	43.4	
4	1.02	0.51	2.00	0.23	1.53	55	35.9	
6	0.76	0.35	2.17	0.19	1.11	53	47.7	
10	1.45	0.56	2.58	0.32	2.01	126	62.6	
11	1.19	0.71	1.68	0.30	1.90	115	62.1	37.3
12	1.21	0.63	1.92	0.30	1.84	118	60.5	
Average..	1.20	0.71	1.81	0.296	1.91	100	51.5	

NUMBER OF DOG	THORACIC DUCT LYMPH							
	Albumin	Globulin	A/G ratio	Lymph/ serum ratio	Total protein	Total osmotic pressure	Osmotic pressure per gram protein	Non-protein nitrogen
	grams per cent	*grams per cent*			*grams per cent*	*mm. H_2O*	*mm. H_2O*	*mgm. per cent*
1	2.63	2.95	0.89	0.73	5.58	229	41.0	34.5
2	2.59	2.01	1.29	0.70	4.60	212	46.0	33.2
3	2.62	1.64	1.60	0.70	4.26	213	50.0	45.0
5	2.10	1.28	1.64	0.55	3.38	164	46.5	39.0
6	2.39	1.55	1.54	0.67	3.94	195	49.4	41.3
7	1.21	0.71	1.71	0.34	1.92	117	60.9	47.6
8	2.49	1.38	1.80	0.66	3.87	157	40.5	
9	2.53	1.87	1.35	0.78	4.40	244	55.4	38.2
10	2.65	1.30	2.04	0.60	3.95	202	51.1	43.0
12	2.44	1.04	2.35	0.57	3.48	163	46.7	33.3
13	3.39	1.25	2.71	0.74	4.64	200	43.1	35.0
Average..	2.45	1.54	1.72	0.65	4.00	191	48.4	39.0

NUMBER OF DOG	LIVER LYMPH						
	Albumin	Globulin	A/G ratio	Lymph/ serum ratio	Total protein	Total osmotic pressure	Osmotic pressure per gram protein
	grams per cent	*grams per cent*			*grams per cent*	*mm. H_2O*	*mm. H_2O*
1	2.78	3.42	0.81	0.81	6.20	212	34.1
5	2.71	1.74	1.55	0.72	4.45	178	40.0
6	3.18	2.38	1.34	0.94	5.56	148	26.6
7				0.90	5.1	159	31.1
Average...	2.89	2.51	1.23	0.84	5.32	174	32.9

TABLE 1—*Concluded*

NUMBER OF DOG	INTESTINAL LYMPH						
	Albumin	Globulin	A/G ratio	Lymph/ serum ratio	Total protein	Total osmotic pressure	Osmotic pressure per gram protein
	grams per cent	*grams per cent*			*grams per cent*	*mm. H_2O*	*mm. H_2O*
5	2.48	1.60	1.53	0.66	4.08	178	43.6
10	2.37	1.52	1.56	0.62	3.89	217	55.7
Average...	2.42	1.56	1.55	0.64	3.98	197	49.6

EXPERIMENTS. The results obtained are shown in table 1.

DISCUSSION. From table 1 it is apparent that lymph from different sources in the same animal has a lower albumin and globulin content than that of the corresponding serum. At the same time the albumin/globulin ratio of the various kinds of lymph is invariably higher. These findings are in accord with those of Wells (1932) for lacteal lymph, and Weech (1933) for leg lymph, and support the contention that the capillary wall is slightly more permeable to serum albumin with its lower molecular weight than to serum globulin.

The total protein content of lymph is invariably lower than that of serum but appears to bear a definite relationship to it as is shown by the lymph/serum ratios. For cervical lymph, the lowest value of the ratio is 0.39, the highest 0.76, the mean, 0.58, and the average deviation from the mean ± 0.083; leg lymph, lowest value 0.19, highest 0.41, mean 0.30, and the average deviation from the mean ± 0.41; thoracic duct lymph, lowest value 0.34, highest 0.78, mean 0.66, and the average deviation from the mean ± 0.74; liver lymph, lowest value 0.72, highest 0.94, mean 0.84, and the average deviation from the mean ± 0.78. These ratios, as Wells (1932) states, "suggest that for a given level of the serum protein concentration the protein content of the lymph may vary only within certain fairly definite limits." This is true not only for lacteal lymph but for lymph from different areas of drainage.

The "specific osmotic pressure," i.e., the osmotic pressure per gram of protein, of serum, and of the different kinds of lymph, with the exception of liver lymph, is very similar. The averages are: serum, 49.1 mm. of water; cervical lymph, 43.7; leg lymph, 51.5; thoracic duct lymph, 48.4; liver lymph, 32.9, and intestinal lymph, 49.6. For the individual experiments the figures for the specific osmotic pressure of lymph may vary, some being slightly above and others slightly below those of the corresponding serum. These figures are in agreement with those of Wells for lacteal lymph and fail to verify previous work by Loewen, Field and Drinker

(1931) in which they found a uniformly higher specific osmotic pressure for lymph than for serum. The only explanation Wells could offer for this discrepancy was that Loewen's figures for the total osmotic pressure did not represent true equilibrium values and were too high by a constant amount.

In view of the fact that Loewen's values for serum were within the range of expectation and agreed with Wells' results, and that his lymph values were obtained in an exactly similar manner, some other explanation seems called for. As previously stated, Loewen's total proteins were determined by the refractometric method. In the case of serum, use was made of the Reiss tables for the conversion of the refractive index into percentages of

TABLE 2

Protein content and colloid osmotic pressure of blood and lymph from dogs (recalculated from Loewen, Field and Drinker, 1931)

DOG	BLOOD SERUM			CERVICAL LYMPH			THORACIC DUCT LYMPH		
	Protein	Osmotic pressure	Osmotic pressure per gram of protein	Protein	Osmotic pressure	Osmotic pressure per gram of protein	Protein	Osmotic pressure	Osmotic pressure per gram of protein
	per cent	*mm. H_2O*	*mm. H_2O*	*per cent*	*mm. H_2O*	*mm. H_2O*	*per cent*	*mm. H_2O*	*mm. H_2O*
1	7.80			4.73	187	40	6.48	286	44
2	7.79	412	53	3.55	172	48	6.60	344	52
							6.05	336	56
3	7.24	371	51	2.72	131	48			
4	7.42	366	49				4.57	214	47
5	6.44	334	52	2.79	160	57	4.49	225	50
6	6.68	466	70	2.97	142	48			
7	3.85			2.50			3.83	181	47
8	7.05	350	50	4.22	195	46	4.92	228	46
9	6.30	340	54	2.74	173	63	3.37	138	41
Average....	6.73	377	54.0	3.27	166	50	5.04	244	47.8

protein. Peters and Van Slyke (1931) state that the refractometric method of Reiss and Van Slyke indicates about 1 gram of total protein per 100 cc. of plasma more than the methods which depend upon the estimation of protein nitrogen. Neuhausen and Rioch (1923) have shown that the divergence is due to an erroneously high factor used by Reiss for converting refractive indices into protein concentration. With a corrected factor the refractometric results for total protein in plasma agreed well with those obtained by Kjeldahl. In the case of lymph no published tables were available, those for transudates and exudates having been found unsuitable, and so conversion tables which had been worked out in this laboratory at the beginning of the work on lymph and which

were based on relatively fewer total nitrogen determinations were used. Since that time a great many more total nitrogen determinations have been made on lymph of all kinds and on edema fluid from dogs with elephantiatic legs. These have all been plotted against refractometric readings of the same material and a curve for our instrument, the Zeiss Dipping Refractometer, constructed (Drinker, Field, Heim and Leigh, 1934). Previous lymph protein values determined by use of the refractometer have all been too low. Loewen's serum protein figures have been recalculated, using the corrected factor for dog serum suggested by Neuhausen and Rioch (1923), and his lymph proteins corrected in accordance with the new tables of Drinker, Field, Heim and Leigh (1934). They agree well with the results reported here for the specific osmotic pressure of serum and lymph and are included in table 2.

From table 1 it can be seen that the total protein content of liver lymph is higher than that of thoracic duct lymph and closely approaches that of the serum. The specific osmotic pressure, however, of the four samples of lymph obtained is considerably lower than that of the serum and of lymph from other sources in the same animal. Of the three cases in which the albumin/globulin ratio of lymph was found to be lower than that of serum, two were in liver lymph and one in the thoracic duct lymph from one of the same dogs. The albumin/globulin ratio of liver lymph more closely resembles that of the corresponding serum than that of any of the other samples of lymph. Until a larger series of measurements is made on liver lymph, we can offer no explanation for the relatively low specific osmotic pressure obtained.

The protein content of leg lymph which was obtained from dogs walking about the laboratory in a wholly normal manner must represent fairly well that of the tissue fluid and the blood capillary filtrate, since under these conditions the opportunity for reabsorption is at a minimum. It will be noted that the protein concentrations are all considerably higher than those previously reported for leg lymph (White, Field and Drinker, 1933). Those figures, however, are in agreement with these obtained by Kjeldahl analyses if corrected in accordance with the new tables. The average "effective osmotic pressure," i.e., the colloid osmotic pressure of the blood serum minus the colloid osmotic pressure of the lymph serum, of the leg region in eight dogs is 203 mm. of water or 14.8 mm. of mercury. The only figure we have available to compare with this is a venous pressure of 14 mm. of mercury in the leg of a normal dog (Drinker and Field, 1933). Since the lymph was collected over a period of two to three hours of continuous walking, it seems reasonable to suppose that most of it represented that which was being newly formed from the blood capillaries at a time when the venous pressure was well over 14.8 mm. of mercury and when reabsorption was consequently negligible.

SUMMARY

1. The protein concentrations and osmotic pressures of serum and lymph from different sources in the same animal have been determined in a series of 13 dogs.

2. The albumin/globulin ratio of lymph is invariably higher than that of the corresponding serum.

3. The total protein content of lymph, always lower than that of serum, appears to bear a fairly definite relation to it as the ratios of lymph protein to serum protein show.

4. The specific osmotic pressure of lymph, with the exception of liver lymph, is similar to that of serum.

5. The "effective osmotic pressure" of lymph obtained from the legs of walking dogs suggests that this lymph must represent fairly well the tissue fluid and capillary filtrate.

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THE LIPIDS OF THE SCLERA, CORNEA, CHOROID AND IRIS

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The nature and the distribution of the lipids of the sclera, cornea, choroid and iris are almost unknown. The only significant chemical fact which has been discovered is that the quantity of cholesterol in the cornea increases with age. This fact, however, has been known for years by the pathologists. The important point which is brought out in this discovery is that the age of the tissues must always be considered in the quantitative analysis of the ocular lipids.

Throughout the ophthalmological literature the information on ocular lipids is frequently confused by the general misconception of the nature of lipids. Commonly all substances which are extractable from sectioned tissue by fat solvents are regarded as true fats. Many times, the phosphorus-containing lipids are classified as lecithin. Birefractive fatty substances are often called cholesterol esters or myelin.

Information on the nature and distribution of the ocular lipids is necessary in order to correct the misconceptions in the literature and to understand the behavior of the lipids in normal and pathological metabolism of the eye.

EXPERIMENTAL PROCEDURE. *Preparation of the tissues.* Normal bovine eyes which were received just after slaughter of the animals were freed from all extraneous tissue. The ocular tissues were immediately dissected by a corps of assistants. The corneas were cut away inside the limbus so as to avoid all scleral tissue. The epithelium and Descemet's membrane with the endothelium were removed from the cornea, leaving the substantia propria. The iris, lens and vitreous humor were then taken out of the eye. The retinal neuroepithelium was completely stripped from the pigmented retinal epithelium. The choroid with the adhering pigmented retinal epithelium was next removed. The iris and choroid with pigmented retinal epithelium were washed with distilled water to remove the blood. After dissecting away all of the extraneous tissue, the sclera was scrubbed with a fine wire brush. The tissues were finely ground mechanically in a mill and the tissue juices were collected with the ground material. From the water content of samples of these tissues before extraction and from the water content of the tissues as reported in previous papers (1, 2, 3),

the proper corrections were made in the calculation of the concentrations of the lipids of these tissues.

Extraction. About 500 grams of moist tissue were used for each extraction. The material was mixed with several volumes of 95 per cent alcohol by stirring. After about 2 hours, the alcohol was rapidly centrifuged off and the alcoholic extraction was repeated again. The tissue was then placed in a large Sohxlet extractor, and extracted with hot alcohol for 5 hours. The process of hot extraction was repeated with fresh alcohol in the same manner until a test sample of the alcoholic solution in the extractor showed no evidence of lipids. The material was mixed well between each extraction.

The unextractable material was determined in the following manner. The extracted tissue was dried in air at 105°C. and weighed. About 100 grams of the dried material were digested with 500 cc. of a solution of 20 per cent sodium hydroxide at 100°C. for 24 hours. The solution of hydrolyzed tissue was then cooled and acidified with hydrochloric acid, after which petroleum ether was added to extract the unsaponifiable substances and fatty acids. Less than 0.5 per cent of their total amount was found to be unextractable. Therefore, the efficiency of the original extraction was at least 99.5 per cent.

Separation of the lipids. The alcoholic solutions were immediately evaporated by vacuum distillation below 40°C. until the alcohol was removed. The residue remaining after the alcohol and water were distilled was repeatedly extracted with petroleum ether. The remaining lipid was extracted with hot benzene, precipitated with acetone, dried and weighed as sphingomyelin and cerebroside.

The petroleum ether solution was evaporated to about 100 cc. The insoluble lipid which separated out was washed with acetone and was added to the sphingomyelin and cerebroside. About 500 cc. of cold acetone were added to the 100 cc. of petroleum ether solution. The precipitate of phospholipids was well washed with cold acetone and then redissolved in petroleum ether. Upon standing, more sphingomyelin and cerebroside separated out and were removed and washed with acetone.

A part of the petroleum ether solution of kephalin and lecithin was evaporated to dryness and then hydrolyzed with a solution of 3.5 per cent hydrochloric acid for 24 hours at 100°C. The amino nitrogen was then determined by the Van Slyke method. The trimethylamine was estimated by the method of Lintzel and Monasterio (4). The amounts of kephalin and lecithin were then easily calculated. From a part of the phospholipid mixture, the kephalin was precipitated by the addition of four volumes of absolute alcohol. The precipitate of kephalin was freed from alcohol and then dissolved in dry petroleum ether. The alcoholic solution of lecithin which remained was evaporated to dryness and then dissolved in dry petroleum ether.

The mixed sphingomyelin and cerebroside fractions were weighed after drying. After hydrolysis of a sample with a solution of 3.5 per cent hydrochloric acid for 24 hours at 100°C., the quantity of galactose was determined. The trimethylamine was estimated by the method of Lintzel and Monasterio (4), and the corresponding amounts of sphingomyelin and cerebroside were calculated.

Lipids of the bovine sclera, cornea, choroid and iris (one year old)

LIPID	SCLERA	CORNEA		CHOROID	IRIS
		Epithelium	Substantia propria		
	(Grams per 1000 grams moist tissue)				
Total lipids	6.224	19.888	2.071	12.803	10.531
Fats (glycerides of fatty acids)	2.680	0.723	0.499	1.897	1.212
Total phospholipids	2.180	15.199	0.593	7.998	7.171
Lecithins	1.170	10.329	0.258	4.476	4.162
Kephalins	0.502	1.093	0.266	2.520	2.341
Sphingomyelins	0.508	3.777	0.071	1.002	0.668
Cerebrosides	0.073	0.661	0.063	0.751	0.343
Cholesterol	0.709	1.952	0.692	1.396	1.328
Carotenoids:					
"Carotene"	0.0016	0.000+	0.0024	0.023	0.034
"Xanthophyll"	0.0008	0.0000	0.00000	0.0000	0.0000
Undetermined (unsaponifiable substances)	0.441	0.939	0.191	0.468	0.194
Free fatty acids	0.120	0.320	0.016	0.200	0.164
Lipids recovered after alkaline hydrolysis of tissue	0.019	0.094	0.015	0.070	0.085

The remainder of the sphingomyelin and cerebroside mixture was extracted with pyridine at 45°C. The solution was cooled to 15°C. and centrifuged. Four volumes of acetone were added to precipitate the cerebroside. The cerebroside was removed by centrifugation, extracted with cold petroleum ether, and dried. Only a slight trace of phosphorus could be detected colorimetrically in the ash of a small sample of cerebroside. After treatment with acetone, the examination of the cerebroside fractions of each tissue by the selenite plate indicated that phrenosin, kerasin and perhaps other cerebrosides were present. The crude sphingomyelin was reprecipitated from the alcoholic solution and petroleum ether by acetone. The precipitate gave a slight color with orcin, showing the presence of a very small amount of cerebroside.

The acetone-petroleum ether solution of fats and other substances was evaporated to dryness after a sample was taken for the determination of cholesterol by the Man and Peters method (5). The residue was hy-

drolyzed with a solution of 10 per cent potassium hydroxide at 100°C. for 4 hours. An equal volume of alcohol was added and the solution was boiled 4 hours. After cooling the solution, the unsaponifiable substances were removed by repeated extractions with petroleum ether. The aqueous solution was then acidified with hydrochloric acid and the solution repeatedly extracted with petroleum ether to remove the fatty acids. The petroleum ether solutions of the fatty acids and unsaponifiable substances were repeatedly washed with distilled water to remove water soluble substances. Cholesterol was also determined on a sample of the unsaponifiable substances.

The "carotin" and "xanthophyll" were determined according to the method of Palmer (6) on samples of the petroleum ether solution of the unsaponifiable substances.

All evaporations were performed in a vacuum distilling apparatus at a temperature below 40°C. The separation of precipitates was made with the aid of a centrifuge. The alcohol was free from aldehydes. The repurified petroleum ether possessed a boiling point of 30 to 60°C. At appropriate intervals, samples of the lipid solutions were taken for weight. The samples were dried in a vacuum at 100°C. to a minimum weight. All procedures were continued without interruption until the lipids were in a solution of petroleum ether completely filling glass stoppered volumetric flasks.

The iodine number was determined by the Hanus method. The iodine number of the scleral lipids was: kephalin 85.7; lecithin, 43.6; fatty acids of glycerides, 55.0; that of corneal struma lipids was: fatty acids of glycerides, 40.6; that of choroid lipids was: fatty acids of glycerides, 128.6; that of iris lipids was: fatty acids of glycerides, 103.6. The sclera saturated fats contained about 10 per cent palmitin and 90 per cent stearin.

Discussion. The distribution of the lipids in the sclera, cornea, choroid and iris is summarized in the accompanying table. The amount of lipids in the sclera varies with the manner of preparation. If extraneous fatty tissue is completely removed with a wire brush, the quantity of lipid is minimal. Since sclera is a typical connective tissue and the corneal substantia propria has a similar nature, it is to be expected that the ratio of fats to the other lipids is high. On the other hand, this ratio is low in the choroid, iris and corneal epithelium. Some of the free fatty acids are lactic acid and other lower aliphatic acids, and others are higher aliphatic acids resulting from decomposition of the lipids.

The amount of phospholipid is partly dependent upon the number of active living cells. In the corneal epithelium and less so in the choroid and iris, the lecithin content is remarkably high. It is natural to assume that lecithin and perhaps kephalin are vitally necessary lipids. A part of the phospholipids of the choroid come from the metabolically active pig-

mented retinal epithelium. It is possible that lecithin in the iris may be the parent substance for the formation of acetylcholine, which under parasympathetic control, activates the iridal muscles. The data show that a large part of the phosphorus-containing lipids in the analysis is not only lecithin, but kephalin and sphingomyelin. The cerebrosides probably partly arise from the nerve fibers.

Relatively large amounts of cholesterol are present in each tissue as free cholesterol. Practically no cholesterol ester was found. The other unsaponifiable substances are chemically unknown. They may be aliphatic alcohols and cholesterol derivatives.

The quantity of carotenoids is minimal. Undoubtedly some of these substances were destroyed by the action of light and by oxidation during manipulation. The presence of "xanthophyll" only in sclera and of the large quantity of "carotene" in the choroid and iris is noteworthy. In the case of carotenoids, "carotene" is to be considered as hydrocarbon carotenoids and "xanthophyll" as hydroxycarotenoids. Other carotenoids may be present. The data on carotenoids are based on the physical separation only, and not on the spectroscopic analysis, on account of the insufficient material.

SUMMARY

The distribution of fats, lecithin, kephalin, sphingomyelin, cerebrosides, cholesterol, and carotenoids has been determined in the sclera, corneal epithelium and substantia propria, choroid and iris.

The material was obtained through Mr. R. L. Fox. The technical procedures were performed with the aid of Mr. W. Tauber.

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THE EFFECT OF THYROXIN ON THE TISSUE METABOLISM OF EXCISED FROG HEART

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There has been much disagreement as to the effect of thyroxin in vitro on excised tissue metabolism. Some workers have reported that thyroxin in vitro increases tissue metabolism (1, 2, 3). Many other workers have found that thyroxin in vitro has no effect on tissue metabolism (4, 5, 6). Since the excised tissue which was used in such experiments does not survive as long as is required for thyroxin to effect an increase in metabolism in vivo (7, 8, 9, 10) it was thought that a tissue which could be kept in vitro in approximately a normal metabolic condition for several days would be most suitable to establish whether or not thyroxin can raise the metabolism of tissue after removal from the organism. Since the excised frog heart will survive and beat for several days under suitable conditions, it was used in the following experiments to answer this question.

The Warburg differential method (11) was used for the determination of the metabolism of the tissue. An excised intact frog heart was used in each Warburg vessel and was prepared as follows: A cannula (10 mm. long) was inserted through the aorta into the ventricle of the exposed heart, and tied in place about the aorta. All the other blood vessels were tied off close to, but without injuring, the sinus. The heart was then severed and suspended by means of the cannula so that the heart, but not the cannula, was immersed in a citrate-Ringer's perfusate[1] whose calcium ion concentration was one millimol per liter. When so prepared, the heart was easily perfused by using a medicine dropper to suck out used, and to inject fresh, perfusate. Between measurements, the hearts were kept at a temperature of about 9°, at which temperature beating practically ceased. When brought into a temperature of 25°, at which their metabolism was measured,

[1] The citrate-Ringer's solution used had the following composition: NaCl, 114 mM/L; $NaHCO_3$, 30 mM/L; KCl, 6 mM/L; $CaCl_2$, 1.5 mM/L; Na_3 Citrate, 0.7 mM/L; dextrose, 0.2 per cent; and phenol red, 0.00075 per cent. The solution was saturated with a gas mixture composed of 5.5 per cent CO_2 and 94.5 per cent oxygen. The frog heart survives well in this solution, although it is isotonic with mammalian plasma rather than with frog plasma.

they soon resumed beating; and comparable hearts were paired together, in the order in which beating was resumed, before the initial determination. A heart with its attached cannula was inserted in each Warburg vessel for measurement of the metabolism. The medium in which the hearts respired in the vessels was of the same composition as that used as a perfusate. Since comparisons were to be made between the metabolism of particular pairs of hearts, from one time to another, the oxygen consumption was calculated as cubic millimeters per hour per pair of hearts.

Ten series of experiments were performed to determine the difference in the effect of perfusion with and without thyroxin over periods of 3, 10, 15, 24 and 42 hours, respectively. Different pairs of hearts were used for each series, and on each pair only two experiments were performed—an initial and a final; for it was found that the more the hearts were handled and shaken, the more quickly they deteriorated. Since the same pair of hearts was used for the initial and final determinations, it was not a matter of significance if the heart pairs differed initially from each other in size and vitality. Before the initial determination every heart was perfused with citrate-Ringer's without thyroxin, in which medium they also respired during the determination. Between the initial and final determinations the thyroxin-treated hearts were kept immersed in thyroxin-containing citrate-Ringer's, whereas the control hearts were kept immersed in citrate-Ringer's without thyroxin. Before the final determination both the control and thyroxin-treated hearts were freshly perfused with their respective citrate-Ringer's, in which medium they also respired during the final determination.

The results of the ten series of experiments have been summarized in table 1. The five odd-numbered series consist of the thyroxin-treated hearts; the five even-numbered series, their respective controls. Series 1 and 2 show that after three hours of perfusion the metabolism of the hearts perfused with thyroxin-containing citrate-Ringer's had fallen off 23 per cent from the initial reading, and that of the control hearts, perfused with citrate-Ringer's without thyroxin, had fallen off 26 per cent. Series 3 and 4 show that after 10 hours of perfusion the metabolism of the hearts perfused with thyroxin had fallen off only 6 per cent, whereas that of the control hearts without thyroxin had fallen off 21 per cent. Series 5 and 6 show that after 15 hours of perfusion the metabolism of the hearts perfused with thyroxin had increased 55 per cent, whereas that of the control hearts without thyroxin had fallen off 20 per cent. Series 7 and 8 show that after 24 hours of perfusion the metabolism of the hearts perfused with thyroxin still showed an increase of 55 per cent, whereas that of the control hearts without thyroxin showed a decrease of 17 per cent. Series 9 and 10 show that after 42 hours of perfusion the metabolism of the hearts perfused with thyroxin was still 52 per cent above the initial, whereas that of the control

hearts without thyroxin was 39 per cent below the initial value. Although the increase in metabolism due to thyroxin was first definitely apparent after 15 hours of perfusion, the fact that the decrease in the metabolism of the thyroxin treated hearts was less than that of the controls after perfusion for 10 hours suggests that the effect of thyroxin was beginning to be exerted at this time. The increase in metabolism noted in these experiments occurred earlier than has been observed in vivo perhaps because the concentration of thyroxin used was much higher than the tissues normally encounter.

Since it was found that thyroxin in vitro increased the metabolism of the

TABLE 1

The effect of thyroxin on the tissue metabolism of excised intact frog heart

SERIES NUMBER	TIME OF PERFUSION	THYROXIN CONCENTRATION	NUMBER OF PAIRS OF HEARTS	MEAN O_2 CONSUMPTION PER PAIR OF HEARTS		
				Initial	Final	Per cent change
	hours	*mgm. per 100 cc.*		*c.mm. per hr.*	*c.mm. per hr*	
1	3	1	16	53.8	41.3	−23
2	3	0	11	93.8	69.2	−26
3	10	1	21	88.8	83.5	−6
4	10	0	10	92.7	73.1	−21
5	15	1	14	53.8	83.2	+55
6	15	0	10	83.8	67.2	−20
7	24	1	17	58.2	90.5	+55
8	24	0	10	54.3	44.9	−17
9	42	1	10	54.0	81.9	+52
10	42	0	20	55.5	33.2	−39

excised intact frog heart, it seemed advisable to determine whether it would have the same effect on sliced tissue under the same conditions. Both sliced frog heart and abdominal muscle of mouse, which can be used without slicing, showed almost no respiration after 20 hours, either with or without thyroxin. Sliced turtle heart survived much better, as its tissue metabolism after 20 hours was still one-third of its initial value; but the decrease was the same with or without thyroxin. Apparently the injury done by slicing under these conditions was too great to allow thyroxin to exert the same effect as in the excised but intact heart.

Whether the observed increase in metabolism of the excised intact hearts was due to increased rate of beat (12, 13, 14) was not revealed by

these experiments, since under the conditions that obtained it was impossible to determine simultaneously the work of the heart and its metabolism.

It is concluded that thyroxin in vitro increased the tissue metabolism of the excised intact frog heart but not of sliced frog heart tissue under the same conditions.

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THE STIMULATION OF PERIPHERAL NERVE-ELEMENTS SUBSERVING PAIN-SENSIBILITY BY INTRA-ARTERIAL INJECTIONS OF NEUTRAL SOLUTIONS

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In a previous communication (Moore, Moore and Singleton, 1934) experiments in intra-arterial injection were reported which indicated that hypertonic solutions of the chlorides of lithium, sodium, caesium, magnesium, calcium and strontium are required to provide stimulation of peripheral nerve-elements subserving pain-sensibility. In contrast, potassium chloride, rubidium chloride and barium chloride were shown to be effective in hypotonic and isotonic concentrations. The present report deals with an additional series of experiments which were performed to determine some of the factors underlying the difference in sensitivity to these two groups of cations.

METHOD. The experiments were performed upon cats anesthetized by the intraperitoneal administration of dial in a dose varying from 50 mgm. per kgm. in large cats to 7.5 mgm. per kgm. in kittens. The salt solutions were tested by injection into the femoral and brachial arteries distal to a ligature at the root of the extremity. The appearance of unmistakable reflex activity, e.g., movements of the extremities, changes in respiration, vocalization, was considered evidence of stimulation of sensory nerve-elements. Solutions which give rise to such stimulation upon intra-arterial injection do not produce similar effects when injected centrally into a vein. Moreover, experiments previously described (Moore, Moore and Singleton, 1934) indicate that the stimulation does not take place while the solutions are confined to the larger arterial trunks. It is assumed, therefore, that the sensory nerve-elements which are affected are located in the periphery, in or about the capillary bed.

The volume of solution injected varied from 2.0 cc. for the femoral artery and 1.5 cc. for the brachial artery in large cats to one-half these quantities in kittens. These volumes are apparently sufficient to displace the blood from the respective arteries, previous experiments having demonstrated that larger injections do not lower the salt concentration required for stimulation.

Unless otherwise indicated, isotonic solutions were used in the study. The isotonic concentration of each salt was determined by calculating the

concentration required to produce the same lowering of the freezing-point as does 0.9 per cent sodium chloride, namely, a depression of 0.526°C. (Landolt-Börnstein, 1923). Thus the following concentrations were determined: 0.64 per cent LiCl, 0.90 per cent NaCl, 1.15 per cent KCl, 1.86 per cent RbCl, 2.64 per cent CsCl, 2.15 per cent $MgCl_2 \cdot 6H_2O$, 1.59 per cent $CaCl_2 \cdot 2H_2O$, 3.77 per cent $SrCl_2 \cdot 6H_2O$ and 2.73 per cent $BaCl_2 \cdot 2H_2O$. These solutions had pH values of 6.5 or above as determined by the glass electrode potentiometer. It has previously been shown that the acidity of solutions injected into arteries must equal that represented by pH 6.0 in order for stimulation to occur as a result of the hydrogen-ion concentration (Moore, Moore and Singleton, 1934). Therefore, any stimulation arising from the solutions listed was not an effect of acidity. Ammonium chloride and beryllium chloride were not included in the series inasmuch as isotonic solutions of these salts had pH values below 6.0.

By combining varying proportions of two isotonic solutions the various series of isotonic mixtures indicated in table 1 were prepared. Each series consisted of graduated combinations of a highly irritating chloride (potassium, rubidium or barium chloride) with a relatively non-irritating chloride (sodium chloride, magnesium chloride, etc.). For each determination the solutions of one series were injected in order of increasing concentration of the irritating component until its threshold for stimulation was reached. A comparison of the threshold for one combination of salts with that for another was obtained by injections into the corresponding arteries of the opposite limbs. Thus it was possible, for example, to compare the action of potassium chloride in association with sodium chloride in one limb with the action of potassium chloride in association with magnesium chloride in the opposite extremity.

The injected material was allowed to return to the general circulation by way of the veins. After each injection the arterial tree was washed with isotonic sodium chloride. In control experiments it was found that injections of the chlorides of the alkaline metals did not modify appreciably the irritability of the reflex arc inasmuch as repeated determinations in one animal gave identical threshold values. In contrast, the salts of the alkaline earth metals, magnesium, calcium and strontium, definitely lowered the sensitivity to subsequent injections. Therefore, in the experiments to be reported, the thresholds for but two mixtures were determined in each animal, all mixtures containing magnesium, calcium or strontium chloride being reserved for the second of the two determinations.

Results. A. *Effects of isotonic solutions on sensory nerve-elements.* Isotonic mixtures of potassium chloride and sodium chloride (table 1, A: (1)) became irritating in most animals when the potassium chloride proportion reached 0.3 x isotonic (0.35 per cent KCl), representing a potassium-ion concentration approximately 0.05 molar (degree of dissociation from

Kraus, 1922). When lithium chloride or caesium chloride was substituted for sodium chloride in such mixtures the threshold at which potassium provided stimulation was not appreciably changed (table 1, A: (2) and (3)).

In the opposite extremities of the same animals isotonic mixtures of potassium chloride with the chloride of magnesium, calcium or strontium were injected. The results, in these cases, presented a sharp contrast. In each of forty-eight animals the presence of the alkaline earth salt resulted in a marked elevation of the potassium threshold (table 1, A: (4), (5) and (6)).

TABLE 1

Occurrence of pseudaffective reactions following intra-arterial injections of isotonic solutions combining varying porportions of two salts

The number of animals in which each combination of salts was tested is indicated in parentheses. The figure in each column represents the number of animals giving a reaction.

SALT MIXTURES TESTED (ISOTONIC MIXTURES OF SALTS x AND y)	PROPORTION OF EACH SALT IN MIXTURE				
	0.1 x + 0.9 y	0.2 x + 0.8 y	0.3 x + 0.7 y	0.4 x + 0.6 y	0.5 x + 0.5 y
A. x = KCl:					
(1) y = NaCl (32 animals)	0	1	24	32	32
(2) y = LiCl (6 animals)	0	2	5	6	6
(3) y = CsCl (8 animals)	0	0	7	8	8
(4) y = $MgCl_2$ (17 animals)	0	0	0	0	0
(5) y = $CaCl_2$ (21 animals)	0	0	0	0	0
(6) y = $SrCl_2$ (10 animals)	0	0	0	0	1
B. x = RbCl:					
(1) y = NaCl (6 animals)	0	1	5	6	6
(2) y = $CaCl_2$ (6 animals)	0	0	0	0	0
C. x = $BaCl_2$:					
(1) y = NaCl (9 animals)	9	9	—	—	—
(2) y = $MgCl_2$ (3 animals)	1	2	—	—	—
(3) y = $CaCl_2$ (3 animals)	0	1	—	—	—
(4) y = $SrCl_2$ (3 animals)	0	2	—	—	—

In another series of experiments hypertonic mixtures of equivalent concentrations of potassium chloride and of magnesium chloride, calcium chloride or strontium chloride were injected to determine the concentration of potassium ion which would serve to provide stimulation in the presence of an alkaline earth salt. In these animals the concentration required for afferent stimulation ranged from 1.6 x isotonic (0.8 x isotonic KCl plus 0.8 x isotonic $MgCl_2$) in some instances to 3.5 x isotonic in others (these experiments are not included in table 1). This variation in threshold was in contrast to the relatively constant values obtained in the experiments with isotonic solutions. Moreover, when stimulation did occur it was not

accompanied by the evidences of direct stimulation of motor elements to be described in the next section as characteristic of potassium injections, and may well have arisen from the hypertonicity of the mixture and not from the potassium content.

Mixtures of rubidium chloride with sodium chloride (table 1, B: (1)) served to stimulate in 5 of 6 animals when the rubidium-chloride proportion reached 0.3 x isotonic (0.56 per cent RbCl), representing 0.04 molar concentration of the rubidium ion. The substitution of calcium chloride for sodium chloride in the solutions injected into the opposite limb of these animals served to elevate the rubidium threshold to at least double this value (table 1, B: (2)).

The cases listed in table 1, C, demonstrate that magnesium, calcium and strontium salts also exerted an antagonism to stimulation by the barium ion. Apparently the presence of a salt of one of these three bivalent alkaline earth cations—Mg, Ca, Sr— interfered in some way with the stimulation of nerve-elements by potassium, rubidium or barium.

B. *Effects of isotonic solutions on motor nerve-elements.* The intra-arterial injection of an isotonic solution of potassium chloride, rubidium chloride or barium chloride resulted in the appearance of one or more of the following evidences of stimulation of afferent nerve-elements: movements of the extremities, dilatation of the pupils, increased rate or depth of respiration, vocalization. In addition to these reflex effects there occurred regularly a gross and rapid twitching of skeletal muscles, confined to the extremity receiving the injection. The twitching began in isolated areas but usually spread to become a generalized quivering throughout the extremity, of sufficient force to produce a rapid clonus at the metatarso- or metacarpophalangeal joints. In some instances this local response endured as long as three minutes. It was not abolished by operative section of all branches of the nerve-plexus at the root of the limb, but was prevented easily by curarization to the stage at which peripheral faradization of motor nerves produced no visible response and spontaneous respiration ceased. It represented therefore, a stimulation of motor nerve-elements.

This direct action upon peripheral motor elements usually became evident at a slightly lower concentration of potassium or rubidium ion than was required to provide sensory stimulation in the same animal. Barium solutions also produced motor stimulation in most cases, but usually at a concentration of barium ion equal to or exceeding that which was sufficient for sensory stimulation.

The addition of an equivalent proportion of magnesium chloride, calcium chloride or strontium chloride prevented the occurrence of this direct motor effect following potassium, rubidium or barium injections, just as it prevented the appearance of reflex signs of sensory stimulation.

C. *Effects of hypotonic solutions upon peripheral nerve-structures.* The

intra-arterial injection of a sufficient quantity of distilled water served to stimulate both sensory and motor nerve-elements just as did injection of an isotonic solution containing potassium, rubidium or barium ion. The reflex effects varied from animal to animal but the local twitching was constant and marked. It was abolished by administration of curare. Three-tenths per cent NaCl produced the same effects as did distilled water. However, the addition to distilled water of sufficient magnesium chloride, calcium chloride or strontium chloride to render the solution 0.1 x isotonic prevented the appearance of both motor and sensory effects. Since addition of three times this amount of sodium chloride did not prevent stimulation, it appears that the alkaline earth chloride also exerted a definite antagonism to irritation by hypotonic solutions.

Discussion. The experiments illustrate that peripheral nerve-structures can be stimulated by neutral solutions of several types. Chlorides of lithium, sodium, caesium, magnesium, calcium and strontium excite pain-elements only when the total salt content of the solution approaches half-molar (Moore, Moore and Singleton, 1934). This irritation is common to all hypertonic solutions which have been tested. In the absence of salts of magnesium, calcium or strontium, hypotonic solutions such as distilled water or 0.3 per cent NaCl serve to stimulate both motor and sensory elements. Similarly, stimulation occurs when the nerve-structures are exposed to isotonic solutions containing certain ions, notably potassium, rubidium or barium. The presence of salts of magnesium, calcium or strontium, however, serves to prevent stimulation of either motor or sensory nerve-elements by hypotonic solutions or by solutions containing the ion of potassium, rubidium or barium.

That a relatively low concentration of magnesium, calcium or strontium ion inhibits the irritation of nerve-structures by hypotonic solutions suggests an interpretation of the facts outlined in the preceding paragraph in terms of the permeability of the cell-membrane. The cell is freely permeable to water (Lillie, 1923), and it is reasonable to assume that hypotonic solutions stimulate the nerve-structure by virtue of the excess water which enters it and gives rise to swelling. According to the permeability hypothesis it would follow that the alkaline earth salts inhibit the passage of this excess water through the nerve-membrane and it is well known that salts of magnesium, calcium and strontium serve to decrease the permeability of the cell-membrane to water (Lillie, 1923; Jacobs, 1924; Lucké and McCutcheon, 1932). Potassium ion and rubidium ion penetrate cells much more rapidly than do sodium and the other ions listed (Lillie, 1923; Jacobs, 1924; Cooper, Dorcas and Osterhout, 1929; Brooks, 1932), and the stimulation of nerve-structures by potassium and rubidium might well be due to the effects of this penetration. The entrance of an excess of the ion into the nerve-structure must disturb the internal ionic equilibrium

and at the same time may cause swelling on account of the water which the ion carries with it. In regard to barium, it may be pointed out that it is more toxic than other alkaline earth ions, producing local irritation and being inimical to lower forms of life (Sollmann, 1932). Any injury to cells results in an increased permeability (Osterhout, 1922, 1923; Lucké and McCutcheon, 1932), and it is suggested that the stimulation by barium may be due to some toxic property which gives rise to cellular injury. On the other hand the analgesic action of magnesium, calcium and strontium salts would be regarded as an expression of the protection they afford the nerve-membrane. Preventing its penetration by water or other agents, the action of these salts resembles the supposed action of narcotics (Lucké and McCutcheon, 1932).

SUMMARY

The sensitivity of peripheral nerve-elements to neutral chloride solutions has been studied by means of intra-arterial injections in animals under dial anesthesia. The results indicate that nerve-structures can be stimulated by neutral solutions of several types. Chlorides of lithium, sodium, caesium, magnesium, calcium and strontium excite pain-elements only when the total salt concentration of the solution approaches half-molar. Moreover, in the absence of salts of magnesium, calcium or strontium, hypotonic solutions such as distilled water or 0.3 per cent NaCl serve to stimulate both motor and sensory elements. Similarly, stimulation occurs when the nerve-structures are exposed to isotonic solutions containing certain ions, notably potassium, rubidium and barium. The presence of salts of magnesium, calcium and strontium, however, serves to prevent stimulation of either motor or sensory nerve-elements by hypotonic solutions or by solutions containing the ions of potassium, rubidium or barium.

These observations suggest that the interpretation of stimulation in terms of the permeability of the cell-membrane (Lillie, 1923) may be extended to apply to the stimulation of peripheral nerve-elements subserving pain-sensibility in the intact mammalian body. Interpreted in this manner, hypertonic solutions stimulate by shrinking the nerve-structures whereas the excitation by hypotonic solutions results from swelling. Low concentrations of those ions which readily penetrate the nerve-membrane, e.g., potassium and rubidium ion, give rise to stimulation. In contrast, ions to which the nerve-membrane is relatively impermeable (lithium, sodium, caesium, etc.) fail to stimulate except in solutions so concentrated as to exert a marked osmotic effect. Salts of magnesium, calcium and strontium, according to this interpretation, play an analgesic rôle by virtue of the protection they afford the nerve-membrane. Inhibiting its penetration by stimulating agents, the action of these salts would resemble that of narcotics.

It has been suggested that under abnormal conditions substances which escape from injured cells into the intercellular fluids may accumulate in concentrations sufficient to affect pain-endings (Lewis and Hess, 1933; Moore, Moore and Singleton, 1934). The results reported at this time indicate that in the identification of such substances the ability to penetrate the cell-membrane may serve as a useful criterion. In view of the demonstration that potassium diffuses from injured cells to accumulate in inflammatory fluids (Häbler and Hummel, 1923; Reimers, 1932), it is of interest that the potassium ion is capable of penetrating nerve-structures and of giving rise to stimulation.

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THE RESPONSE OF THE CANINE AND HUMAN PANCREAS TO SECRETIN

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For the past five years we have been endeavoring in this laboratory to purify secretin to the point where its use in human patients would be without danger. The ultimate purpose of this research is to perfect a pancreatic function test, if practical, using secretin stimulation much in the same manner as histamine is now used in stimulation of the gastric glands.

A method of preparing this product has evolved which yields a secretin of potent quality. Using our secretin preparation, 22 apparently normal human subjects have been subjected to the intravenous injection of this substance. No known pathological conditions were investigated in this preliminary series which was designed to form the basis of a report on the response of the normal human pancreas to the intravenous injection of a standard dose of secretin. However, before studies were conducted on the human, a number of pertinent experiments were performed on dogs.

The only significant literature on this subject is that published by Chiray and his collaborators. Chiray, Salmon and Mercier in 1926 (1) used a purified secretin, free from hypotensive effect and with but little cholecystokinetic activity, in doses of 10 to 30 mgm. (total dose) by intravenous injection into human subjects. They observed regularly an increase in the rate of flow of fluid from the duodenal tube after the injection of this material, and an increase in the concentration of lipase and trypsin in the duodenal drainage. In this and subsequent work by Chiray, Jeandel and Salmon in 1930 (2), routine evacuation of the gall bladder by intraduodenal instillation of concentrated magnesium sulphate was practiced before stimulating the pancreas with secretin. The "C bile" was used as the control sample. These latter authors using the same preparation of secretin, investigated the response of the pancreas to a standard secretin dose in a number of normal and pathological cases. The potency of their secretin preparation was not stated in terms that may be interpreted in units or animal doses. They observed in their normal

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subjects an increased flow of duodenal fluid from the Einhorn tube following secretin injection and a two to threefold increase in the concentration of pancreatic enzymes as compared to the "C bile" controls. In those patients with pancreatic lesions or associated pathology, this characteristic increase in flow or enzyme concentration did not occur or was not marked in 17 out of 29 cases. In repeated trials on the same patient, comparable results were obtained for each individual determination.

METHODS. *Preparation of secretin.* Crude secretin is prepared by extracting hog duodena with 0.4 per cent hydrochloric acid for one-half hour and saturating the extract with sodium chloride. The precipitate (A) contains most of the secretin and in this form may be kept for months in the ice-box without deterioration. Further purification is obtained by alcoholic extraction of this precipitate and vacuum distillation (water bath at 40°, solution in flask about 20°) of the extract. A watery suspention of the residue is brought to a pH of 5.4, filtered and the filtrate treated with sufficient trichloracetic acid to make a concentration of 5 per cent of the acid. This precipitates a potent secretin (S-I) which, when dried with a mixture of acetone and ether, is stable and easily soluble in a dilute acid solution. The preparation of secretin up to this point has been fully discussed in previous publications (3) (4). The final purification which was developed by one of us (H. G.) will be discussed in detail.

Five hundred milligrams of finely pulverized secretin (S-I) are dissolved in 10 cc. of redistilled water plus 0.1 cc. of concentrated hydrochloric acid. Acetone (C. P.) is added until the precipitate which forms fails to redissolve on stirring or until four volumes of acetone have been added. Ten cubic centimeters of aniline, which has been redistilled over tin and which is colorless, are added with stirring and the precipitate is allowed to flocculate. If four volumes of acetone have not been added up to this point, the remainder is now added. Centrifuge at moderate speed for five minutes and decant the supernatant liquid into a 500 cc. distilling flask. Wash the precipitate once with a mixture of acetone, aniline and water in the proportions obtaining in the precipitating solution, adding the washings to the flask. The acetone is now distilled off under vacuum without heat. A water bath at 30° is then placed around the distilling flask and the aniline is removed by vacuum, adding water from time to time to keep the volume constant to about 100 cc. When the last of the aniline has been removed, as is shown by the absence of droplets of aniline on the walls of the receiver, the solution is taken to dryness at 30° and allowed to stand under vacuum for one-half hour. The residue in the flask is washed out with four 10 cc. portions of absolute methyl alcohol, centrifuged and the supernatant fluid transferred to a 250 cc. flask. Precipitation of the secretin is accomplished by the addition of five volumes of anhydrous ether. The precipitate is collected by centrifuging and dried in a desiccator.

Secretin in the dry form is stable for indefinite periods. It is rapidly inactivated in neutral or alkaline solution. In acid solution of pH 4.0 it is stable for 24 to 48 hours. The product is a colorless, amorphous solid, possessing a bitter taste, readily soluble in water and dilute acids or alkalies. Tests for the elements show the presence of carbon, hydrogen, nitrogen and chlorine. The chlorine is undoubtedly present as a hydrochloride, since a precipitate is obtained with silver salts without destroying the organic matter. The presence of sulphur could not be demonstrated either with barium salts or nitroprusside, after either wet or dry oxidation. Mention is made of this, since cystine has been reported present in the cruder samples. The material chars to a tarry mass at about 160°. The nitrogen content, by the micro-Kjeldahl method, is 6.9 per cent. Contamination of the product with aniline is ruled out by a negative potassium bichromate test.

For injection purposes, we believe it to be advisable to prepare the secretin solution fresh by dissolving the required amount in normal saline and filtering through a Berkefeld filter which has been used for no other purpose and which is washed thoroughly after each filtration.

Animal experimentation. As a preliminary to work on the response of the human pancreas to secretin, the subject was investigated in a number of dogs with and without anesthesia. In the former group of dogs, the effect of extirpation of portions of the pancreas was noted in regard to the response to a standard dose of secretin. A cannula was placed in the major pancreatic duct and a control response from the intact pancreas was determined, using a dose of one milligram of secretin. A second and third response was then elicited to the identical dose after removal of the head or tail of the pancreas or both. The unanesthetized series of dogs consisted of four animals in which a permanent fistula of the duodenum had been created and time for complete healing had elapsed. After 24 to 48 hours of starvation, a catheter was inserted into the duodenum and the control flow determined. An injection of secretin was then made and the rate of flow again determined, both immediately after injection and again after an interval of twenty minutes. Analysis of all samples for bilirubin, cholesterol and the pancreatic ferments (amylase, lipase and trypsin) was made.

Human investigation. In determining the response of the human pancreas to secretin, normal students were used as subjects. They were instructed to eat only a light carbohydrate meal the evening before the test and to abstain from breakfast. The duodenal tube was passed, and its position checked by fluoroscopy in those subjects in which the position of the tube was in question. The drainage from the duodenum immediately after intubation was usually rather rapid for several minutes. After an interval of from 30 to 60 minutes, the flow from the tube usually decreased to what we term a "basal" rate, for we have observed that this slow rate of drainage is not augmented during a two to three hour period of time

if the duodenum, pancreas or gall bladder are not stimulated. Control samples were collected during this period. After the collection of several control samples, an intravenous injection of 15 mgm. of secretin (300 threshold dog doses or units) was made. The first 3 subjects received 25 mgm. of secretin but this amount was reduced to 15 mgm. as it was felt that this latter quantity might allow the recognition of minor variations more readily than when the larger dose was used. Collection of samples after the injection was the same as during the control period, collecting flasks being changed usually whenever a marked change in the volume or character of the flow was noted. This was done to ascertain the maximum rate of flow, which we were interested in from an academic viewpoint. Collection was continued for one-half hour after the injection. The gall bladder was not evacuated prior to the test because the secretin preparation used by us in the human work possessed only a little or no cholecystokinin and we wished to avoid a possible stimulation of the pancreas during the control period or a dilution of the control samples with magnesium sulphate solution or with an augmented amount of intestinal juice or hepatic bile.

Chemical analyses of the duodenal contents included determinations of biblirubin (van den Bergh method), cholesterol and enzyme analysis. Amylase was determined by both the Wohlgemuth (5) and Willstätter (8) methods, lipase by the McClure method as modified by Cherry and Crandall (6) and trypsin by the method of Willstätter (7). In addition, the specific gravity of each sample was determined by the pyknometer method. Enzyme samples should be placed in the ice-box immediately after collection. Apparatus should be prepared before the test so that the analysis of these enzymes may be determined immediately after the test is completed.

For convenience in this and future work, the units by which the enzyme determinations are reported have been standardized. Amylase and trypsin determinations are both reported in standard Willstätter units (7) (8). Lipase is reported in units representing the number of cubic centimeters of 0.1N NaOH required to neutralize the fatty acids liberated by 1 cc. of the undiluted enzyme solution.

The cholesterol was determined by heating from 2 to 15 cc. (depending inversely on the amount of bile pigment present) of the duodenal drainage in a small beaker with 2 cc. of 2 per cent KOH in 95 per cent alcohol on a water-bath, evaporating to a small volume, and drying this on a small piece of loosely rolled absorbent cotton, never omitting to wash thoroughly the beaker and similarly dry the washings. The cotton is then extracted with petroleum ether for one and one-half hours in a continuous extractor of any desired type. A convenient apparatus for this is a 15 x 1 cm. test tube open at the bottom and with a constriction about 2 cm. from the bottom, contained in a larger test tube; for a condenser we have used an

ether can as a common water jacket for any desired number of glass tubes which may be passed through it. The petroleum ether extract is evaporated to dryness, the residue taken up in chloroform, and the cholesterol determined colorimetrically in the usual way.

For the sake of simplicity, certain modifications have been made in the Willstätter methods for trypsin and amylase, which are designed to render them of general applicability to samples of duodenal drainage fluid. These will be described briefly elsewhere.

RESULTS. *Animal experiments.* The effect of repeated partial extirpation of the pancreas in barbitalized dogs is to cause a progressive diminution in the total response of the remaining pancreatic tissue to a standard dose of secretin. The results in three dogs were so uniformly similar to those tabulated in table 1 that it was thought unnecessary to investigate the question further.

The results would indicate that, per gram weight, the head of the pancreas is the most active in responding to secretin, the tail of the pancreas is

TABLE 1

Showing the response of the pancreas to secretin before and after partial extirpation of the gland

PANCREAS REMOVED	PANCREAS REMAINING INTACT	DOSE	RESPONSE
Normal	Entire gland (42.8 gm.)	1 mgm. S-1	4.3 cc.
Head (15.2 gm.)	Body and tail (27.6 gm.)	1 mgm. S-1	1.5 cc.
Tail (8.8 gm.)	Body (18.8 gm.)	1 mgm. S-1	0.7 cc.

less responsive and that the body of the pancreas, considering its bulk, is relatively inert.

This conclusion is justified by a consideration of the fact that the removal of a certain number of grams of the pancreas in the region of the head of the gland decreases the response to secretin considerably more than does the removal of an equal weight from the region of the tail of the gland. This relation holds regardless of whether the tail or the head of the pancreas is removed first. Further, the response of the pancreas to a standard dose of secretin is apparently dependent upon the percentage of functional acinar tissue available after partial removal of the gland; there is no evidence of an immediate compensatory hypersecretion of the tissue remaining in an endeavor of the incomplete gland to respond maximally to a standard dose of secretin. The foregoing is of great importance in the consideration of a pancreatic function test utilizing secretin as a stimulating agent. The fact that the head of the gland appears to be the most active portion is also of importance since pancreatitis and pancreatic disease usually involve this region of the pancreas.

Partial extirpation of the pancreas (head, tail or both) in dogs with a duodenal fistula invariably resulted in a marked decrease in response to secretin and an inability to collect samples from the duodenum that were large enough for a complete enzyme analysis, both before and after secretin stimulation. This marked decrease even persisted for as long a period as two months, indicating little or no functional regeneration of the gland.

In the series of four duodenal fistula dogs with the pancreas intact, in which the pancreatic response to secretin was determined in a total of 15 trials, an experiment comparable to the later work on humans was performed. In this series of dogs a secretin preparation was used which contained considerable cholecystokinin. Following a latent period of from 15 seconds to three minutes after injection the color of the duodenal drainage always became much darker indicating an evacuation of the gall bladder. Bilirubin and cholesterol values were always increased in the post-injection samples. Bilirubin values as high as 602 mgm. per cent and cholesterol values as high as 102 mgm. per cent indicated gall bladder evacuation. The flow from the fistula was always increased after secretin injection. In view of the fact that certain preparations of secretin regularly increase the motor activity of the duodenum, it is thought that the augmented flow may be in part due to this factor; however, the total volume drained from the duodenum is always much greater after secretin injection than during the control period.

Analysis of the enzyme content of control samples, as compared to samples collected immediately after injection and again after an interval of 20 minutes after injection, showed in view of the results of Chiray and his co-workers (1) (2), a surprising lack of uniformity in enzyme concentration. For convenience in nomenclature in this series of determinations, the control sample is called "A," the portion collected immediately after the injection is called "B" and the final sample, collected twenty minutes after injection is called "C." As may be seen from the summary of data presented in table 2 in 15 consecutive trials the amylase concentration of the duodenal fluid immediately after injection of secretin (sample B) was increased over the concentration found in the control sample in seven instances; it was decreased in the remaining eight. In sample C, collected 20 minutes after injection, the amylase concentration was found to be higher than the control sample in only 4 instances and was less than the control in 11. The lipase concentration in sample B was increased over the control in 12 cases and was decreased in only 1. In sample C the concentration of this enzyme was greater than the control sample in 9 trials and less in 5. The determination of trypsin concentration was remarkable in that this enzyme showed a lower concentration immediately following the injection of secretin (sample B) than was present in the control sample in

nine out of the 15 trials. In sample C the concentration was lower than the control drainage in 12 out of the 15 trials.

In view of the literature (1) (2) cited, these results were somewhat disappointing. Chiray noted a definite increase in the enzyme concentration of the duodenal drainage following secretin injection and further reported comparable results in different tests of the same individual. In dogs with the pancreatic duct cannulated, most workers (9) report a decrease in the concentrations of enzymes in the pancreatic juice following secretin stimulation (vide infra). In our work on dogs we were unable to detect any constant increase in the concentration of enzymes in the duodenal drainage following the injection of secretin. This discrepancy may in part be explained by a consideration of the fact that in this work on dogs the gall bladder was allowed to evacuate during the collection of post-injection samples. This dilution of the pancreatic secretion with bile could hardly explain by itself the lack of a uniform increase in enzyme

TABLE 2

Showing the incidence of increase or decrease in enzyme concentration after secretin injection as compared to the control sample in 15 trials on 4 dogs. Sample B immediately after injection. Sample C 20 minutes after injection

	SAMPLE B		SAMPLE C	
	Increased	Decreased	Increased	Decreased
Amylase	7 tests	8 tests	4 tests	11 tests
Lipase	13 tests	2 tests	9 tests	6 tests
Trypsin	6 tests	9 tests	3 tests	12 tests

concentration, since a decrease in this concentration after secretin injection was not always accompanied by evidence indicating a marked evacuation of the gall bladder. Conversely an increase in the concentration of enzymes often occurred in the presence of evidence denoting a powerful and complete evacuation of the gall bladder. Further, comparable results were not obtained in several tests on the same animal. In this connection, a careful analysis of the data collected on these animals has yielded no facts which allow a correlation of the interrelation of the concentration of the individual enzymes to each other. Thus, while an *increased* concentration of any one or more of the enzymes might occur following secretin injection, a simultaneous *decrease* in the concentration of the remaining enzymes might also occur, or vice versa. This was the case in 53 per cent of the fifteen trials. In only 8 instances was the concentration of all of the enzymes either simultaneously raised or lowered, and in these 8 instances the degree of change was not the same for each enzyme (table 3).

It should be emphasized that the foregoing figures represent the concen-

tration of enzyme per unit volume. When the total enzyme production per unit time is considered, it is seen that an increase in enzyme production always follows secretin injection (table 3). Thus, in one minute, the

TABLE 3

Showing the results in the dog of enzyme analysis of duodenal contents before and after the injection of secretin

The time indicates the period in minutes necessary to collect a 5 cc. sample. Sample B is collected immediately after injection. Sample C collected 20 minutes after injection. Enzymes shown in units per cubic centimeter.

DOG NUMBER	TEST NUMBER	SAMPLE	SPECIFIC GRAVITY	BILIRUBIN	AMYLASE	LIPASE	TRYPSIN	TIME
								minutes
I	1	A	1.0191	49.4	0.187	14.2	2.66	25
		B	1.0401	259.0	0.168	14.2	1.43	1
		C	1.0118	23.8	0.162	14.2	1.24	3
	2	A	1.0099	7.5	0.174	36.0	1.14	20
		B	1.0311	176.0	0.170	46.0	1.95	0.5
		C	1.0255	126.0	0.184	40.0	1.86	15
	3	A	1.0157	38.8	0.204	36.0	1.98	25
		B	1.0381	257.0	0.224	38.4	2.03	0.5
		C	1.0119	33.8	0.195	22.4	1.02	10
	4	A	1.0189	86.5	0.198	100.0	1.89	30
		B	1.0341	263.2	0.178	84.8	1.39	3
		C	1.0214	110.4	0.169	68.0	1.44	10
II	1	A	1.0099	4.5	0.152	10.4	1.43	25
		B	1.0237	148.0	0.202	74.0	1.48	1
		C	1.0161	18.9	0.209	98.4	2.59	3
	2	A	1.0140	16.1	0.216	28.0	2.81	20
		B	1.0400	264.0	0.187	52.0	1.51	1
		C	1.0191	23.5	0.308	82.4	2.34	5
III	1	A	1.0138	29.6	0.190	Inactive	2.44	15
		B	1.0355	301.7	0.185	226.0	2.30	0.5
		C	1.0129	31.8	0.175	48.8	1.35	5
	2	A	1.0104	23.5	0.177	14.2	1.29	30
		B	1.0419	357.0	0.232	104.8	1.76	0.5
		C	1.0135	36.9	0.172	33.6	0.257	15
	3	A	1.0127	28.0	0.258	64.0	2.39	10
		B	1.0437	602.0	0.276	172.0	1.69	0.25
		C	1.0146	103.9	0.207	56.0	1.40	2
	4	A	1.0166	26.4	0.263	41.6	1.95	25
		B	1.0396	143.3	0.239	251.2	1.71	1
		C	1.0101	31.5	0.214	99.2	1.43	2
	5	A	1.0155	30.0	0.205	5.6	2.06	15
		B	1.0360	264.0	0.303		3.01	0.25
		C	1.0129	41.8	0.188	113.6	2.32	3

TABLE 3—*Concluded*

DOG NUMBER	TEST NUMBER	SAMPLE	SPECIFIC GRAVITY	BILIRUBIN	AMYLASE	LIPASE	TRYPSIN	TIME
								minutes
IV	1	A	1.0074	3.5	Inactive	Inactive	Inactive	25
		B	1.0153	80.9	0.181	12.0	0.886	2
		C	1.0136	30.1	0.172	29.0	0.790	4
	2	A	1.0151	56.4	0.203	86.0	2.52	10
		B	1.0365	308.0	0.198	123.6	2.57	0.5
		C	1.0155	72.6	0.180	60.0	1.23	3
	3	A	1.0217	60.0	0.273	126.0	2.19	15
		B	1.0381	141.9	0.241	224.0	2.09	1
		C	1.0065	17.8	0.177	74.4	1.35	2
	4	A	1.0131	9.2	0.200	2.4	2.79	32
		B	1.0332	112.0	0.467	72.0	2.32	1.5
		C	1.0132	17.8	0.158	21.6	2.40	3

amylase elaborated by the pancreas immediately after the injection of secretin is from 5 to 158 times as much as was produced in an equal period of time during the control flow. The production of amylase during the collection of "sample C" is from 2 to 41 times as fast as during the control period. The total amount of lipase secreted by the pancreas immediately after secretin injection is enormously increased over the control period, being increased from 9 to 420 fold. Twenty minutes after the injection (sample C) the lipase production was still from 1½ to 80 times as much as during the control period. Total trypsin output was increased from 7 to 92 times immediately after secretin injection as compared to the control period. Twenty minutes after injection, the trypsin output was still from 2 to 15 times as much as in the control period in 14 instances. In only one case did the total output of trypsin per minute fall below that of the control period and in this one instance not until 20 minutes after the injection. The average increase in total enzyme output after secretin injection as compared to the control period is as follows: *a*, sample B, immediately after injection, amylase, 56 times; lipase, 100 times; trypsin, 37 times; *b*, sample C, 20 minutes after injection, amylase, 6 times; lipase, 20 times; trypsin, 4 times.

Specific gravity determinations showed a uniform increase in sample B. This is, of course, due to the presence of gall bladder material of high specific gravity in the duodenal contents. The specific gravity of sample C was usually as low or lower than the control sample.

As was mentioned earlier, investigators working on anesthetized dogs have reported that the pancreatic juice elaborated in response to secretin is poorer in enzymes than is the fasting secretion. *This fact is the keystone in the conception of a pancreatic function test utilizing secretin and determines*

whether analysis of the duodenal drainage shall be interpreted in terms of enzyme concentration or in terms of total enzyme output.

In order to examine this fact in the unanesthetized dog, a series of 5 animals were anesthetized and operated upon, a pancreatic fistula being made. The fistula was maintained in a sterile condition and the animal

TABLE 4

Showing the characteristics of pancreatic juice drained from pancreatic fistula dogs before and after secretin injection

Enzymes shown in units per cubic centimeter

DOG	SAMPLE	SPECIFIC GRAVITY	LIPASE	TRYPSIN	AMYLASE
I	Fasting	1.0144	116.8	2.13	0.189
	Secretin	1.0161	200.4	1.43	0.158
II	Fasting	1.0102	180.0	1.81	0.179
	Secretin	1.0131	172.0	Inactive	0.172
	Fasting	1.0041	154.0	1.81	0.179
	Secretin		152.8	1.43	0.172
III	Fasting	1.0091	52.0	2.05	0.212
	Secretin	1.0121	12.0	1.14	0.199
	Fasting	1.0155	206.0	2.22	0.430
	Secretin	1.0139	158.0	1.43	0.258
IV	Fasting	1.0106	144.0	1.68	0.182
	Secretin	1.0162	172.4	1.79	0.194
V	Fasting	1.0108	98.4	0.457	0.167
	Secretin	1.0156	174.4	0.857	0.208
	Fasting	1.0101	34.4	1.19	0.148
	Secretin	1.0156	68.8	1.09	0.188
	Fasting	1.0088	56.0	0.685	0.608
	Secretin	1.0152	99.2	1.02	0.260
	Fasting	1.0133	218.0	1.50	0.952
	Secretin	1.0143	84.0	1.67	0.738

was sacrificed with ether upon the first indication that sterility had not been maintained. Following a period of fasting a sample was collected from the fistula for an analysis of the "fasting secretion." Immediately after the collection of this sample, the animal was injected intravenously with secretin and a second sample obtained for analysis of the "secretin secretion." From analyses of these samples, which are presented in table 4,

it may be seen that the concentration of enzymes in the control (fasting) sample is usually higher than in the sample obtained after the injection of secretin. This was the case in 50 per cent of the determinations made on the lipase concentration. However, the trypsin concentration is higher in the control than in the secretin juice in 60 per cent of the cases and the amylase concentration is greater in 70 per cent of the cases. The concentrations of all three of the enzymes were simultaneously increased in only two of the ten trials while the concentration of all of the enzymes was simultaneously decreased in only four of the ten trials. In the remaining four trials, some of the enzyme concentrations went up while others went down. In those in which all three of the enzyme concentrations either increased or decreased simultaneously, they were not changed to the same degree. Specific gravity determinations usually showed an increase in the secretin juice as compared to the starvation secretion.

This differential elimination of enzymes after secretin stimulation of the pancreas suggests that the difference is due to differences in the quantity of the enzymes stored or preformed in the cells.

Recapitulation of the results obtained in the experimental work on dogs justifies the following conclusions: 1. Repeated partial extirpation of the pancreas in barbitalized dogs causes a progressive diminution in the amount of pancreatic juice secreted in response to a standard dose of secretin. In unanesthetized duodenal fistula dogs it was impossible to obtain adequate samples (only 1 or 2 cc.) for analysis from the duodenum after the removal of only one-third to one-half of the pancreas. This persisted for two months and probably indicates some "traumatic pancreatitis," although histological studies were not made. 2. In duodenal fistula dogs with the pancreas intact, an injection of secretin always caused a markedly increased flow of duodenal contents from the fistula. *The concentration of enzymes in this drainage material was not consistently higher than the concentration seen in the control samples before injection. The total output of enzymes per unit time was always increased during the 20 minutes post-injection period,* when compared to the total secretion in an equal period of time during the control flow. This is, of course, explained on the basis of a much more rapid flow of the duodenal drainage after secretin stimulation. 3. The production of the individual enzymes by the pancreas is not stimulated to an equal degree by secretin injection. Thus, the production of any one enzyme may be greatly increased over the production of the remaining enzymes. 4. In general, it appears that secretin stimulates the output of lipase to the greatest degree. Amylase is increased to a lesser extent and trypsin is least affected, although such generalization is not invariably true. 5. The effect of secretin stimulation is decreased but still prominent as long as 20 minutes after injection. 6. The total output of enzymes by unanesthetized pancreatic fistula dogs is always increased after

secretin. However, the concentration of enzymes in the pancreatic juice after secretin stimulation may be raised or lowered in approximately an equal number of instances. Changes in the concentration of the individual enzymes is never the same in any one test.

Investigation on human subjects. Little difficulty was experienced in getting the Rehfuss tube in good position in the duodenum. Immediately upon passing the pylorus, the tube began to deliver an alkaline fluid (litmus paper test used), which was transparent and nearly always tinged with bile. In only two instances out of the series of 22 human subjects was this control drainage entirely free of bile. In nearly every case during the collection of control samples the drainage became turbid and acid in reaction for short intervals. We interpret this reaction as being due to an opening of the pylorus and the admission of stomach contents into the duodenum. Upon alkalinization of these samples they rapidly became clear and resembled the other control samples. They were rejected for enzyme analysis, however, because of the unknown factor of dilution by gastric contents which had been introduced. It is probable that if the stomach had been constantly drained during the experiment this reaction would not have occurred.

Physical characteristics of the duodenal drainage fluid after secretin injection. Little or no color change from the pale brown of the control samples was noted following the injection of secretin. This is contrary to our results in the preceding animal experiments. The difference lies in the fact that in the animal work a secretin preparation was used which contained considerable cholecystokinin whereas in the human work the secretin used was practically free from this substance. Specific gravity determinations (table 5) on all samples show little or no significant increase after secretin injection.

Minute flow of drainage. The drainage from the duodenum immediately after intubation was usually rather rapid for several minutes. It soon reached a "basal" rate of flow, however, during which period the control samples were collected. The rate of flow during this basal period varied from 0.2 to 3.7 cc. per minute with an average of 1.16 cc. per minute for the twenty-two trials (table 6).

Following an injection of secretin the rate of flow from the Rehfuss tube was always increased. The period of maximum flow started in about half of the cases before the injection (which usually covered about 3 minutes) was finished. In the others it was delayed as long as from 2 to 13 minutes. The average latent period was 3.3 minutes. The maximum rate of flow of fluid after secretin injection varied between 3 and 44 cc. per minute with an average for the 22 trials of 10 cc. per minute (table 6). In six of the 22 subjects a secondary peak in the post-injection flow was noted which occurred in from 7 to 25 minutes following the first period of maximum

flow. In view of the rarity of this secondary peak and its lack of uniformity we do not believe it to be evidence of a biphasic response of the pancreas to the injection of secretin.

The average, minimum and maximum rates of flow during the 15 and 30 minute periods after the injection are shown in table 8. It is to be noted that the response of two of the subjects, namely, nos. 10 and 17, was abnormally low. These two subjects have free fat in the stools, but their

TABLE 5

Showing the concentration of bilirubin and cholesterol and the specific gravity of the duodenal contents before and after secretin injection (human)

SUBJECT	BEFORE INJECTION			AFTER INJECTION		
	Specific gravity	Bilirubin	Cholesterol	Specific gravity	Bilirubin	Cholesterol
		mgm. per cent	*mgm. per cent*		*mgm. per cent*	*mgm. per cent*
1	1.0070	4.1	Trace	1.0071	55.5	22.6
2	1.0040	45.0	11.3	1.0050	26.1	7.8
3	1.0049	4.9		1.0088	3.8	2.8
4	1.0107	None	None	1.0095	None	None
5	1.0071	10.5	9.2	1.0094	17.5	15.6
6	1.0071	11.7	10.8	1.0075	4.7	13.2
7	1.0088	15.0	29.2	1.0076	13.7	24.1
8	1.0067	5.5		1.0089	Trace	
9	1.0086	25.8	18.7	1.0095	22.9	17.1
10	1.0089	3.1	3.6	1.0096	Trace	None
11	1.0097	8.7	8.6	1.0111	34.0	29.8
12	1.0064	2.7	Trace	1.0088	32.7	25.6
13	1.0104	129	315	1.0096	91.0	186
14	1.0092	48.1	70.6	1.0109	43.8	63.7
15	1.0083	35.5	61.7	1.0105	35.8	63.5
16	1.0087	23.9	33.2	1.0146	34.3	42.7
17	1.0074	16.3	21.2	1.0086	15.0	20.0
18	1.0080	30.8	36.5	1.0111	40.0	51.2
19	1.0101	45.4	56.6	1.0108	35.3	43.8
20	1.0041	None	None	1.0145	50.8	61.7
21	1.0075	7.8	12.5	1.0087	16.5	30.6
22	1.0071	18.7	22.4	1.0091	22.5	28.0

general nutrition is good. Thus, in our opinion, a normal subject after the injection of *the dose of secretin used* should show a response of at least 3.3 cc. per minute during the first 15 minutes and 2.0 cc. per minute during the first 30 minutes after injection. The total flow of fluid for the 15 minute post-injection period should be increased at least 2.5 times approximately over that of the 15 minute pre-injection or control period.

Bilirubin and cholesterol. Determinations of bilirubin and cholesterol in the duodenal drainage were made in order to ascertain whether or not an

TABLE 6

Showing the total output of enzymes before and after secretin

Enzymes given in average unit outputs per minute and in total unit output for 15 minute control and 15 minute and 30 minute post-injection periods.

SUBJECT	PERIOD	CUBIC CENTIMETERS PER MINUTE	TRYPSIN		AMYLASE		LIPASE	
			Units per minute	Total	Units per minute	Total	Units per minute	Total
1	Control	0.5	1.19	17.85	0.173	2.595	45	675
	Post-injection							
	15 minutes	3.3	6.48	97.20	0.412	6.180	185	2,775
	30 minutes	3.3	5.98	179.4	0.232	6.960	116	3,480
	Maximum	5.0	9.53		0.452		300	
2	C	1.2	2.76	41.4	0.178	2.670	75	1,125
	15	5.2	9.24	138.6	0.466	6.990	464	6,960
	30	2.9	6.78	203.4	0.261	7.830	397	11,910
	Max.	7.7	14.80		0.941		662	
3	C	0.5	0.24	3.6	0.059	0.885	4	60
	15	4.9	3.43	51.5	0.215	3.225	238	3,570
	30	3.4	2.40	72.0	0.192	5.760	163	4,890
	Max.	44.0	38.2		1.225		2,830	
4	C	0.2	0.48	7.20	0.153	2.295	50	750
	15	4.5	6.24	93.6	0.462	6.930	770	11,550
	30	3.6	3.46	103.8	0.249	7.470	466	13,980
	Max.	5.2	8.36		0.506		1,104	
5	C	0.5	0.69	10.35	0.164	2.460	51	765
	15	4.9	6.26	93.9	0.267	4.005	777	11,655
	30	4.5	3.42	102.6	0.170	5.100	451	13,530
	Max.	7.5	23.7		0.892		1,096	
6	C	1.8	1.92	28.8	0.205	3.075	85	1,275
	15	4.8	7.89	118.3	0.488	7.320	287	4,305
	30	4.0	4.13	123.9	0.257	7.710	151	4,530
	Max.	6.7	12.38		1.315		370	
7	C	1.4	0.72	10.8	0.302	4.530	Trace	
	15	5.0	4.48	67.2	0.209	3.135	268	4,020
	30	5.9	4.93	147.9	0.201	6.030	584	17,520
	Max.	9.0	9.75		0.512		954	
8	C	2.1	0.525	7.875	0.184	2.760	65	975
	15	4.7	3.09	46.35	0.332	4.980	264	3,960
	30	3.4	1.88	56.40	0.178	5.340	274	8,220
	Max.	5.8	3.32		1.118		288	

TABLE 6—*Continued*

SUBJECT	PERIOD	CUBIC CENTIMETERS PER MINUTE	TRYPSIN		AMYLASE		LIPASE	
			Units per minute	Total	Units per minute	Total	Units per minute	Total
9	C	0.4	0.46	6.90	0.164	2.460	37	555
	15	3.6	3.56	53.4	0.211	3.165	433	6,495
	30	3.2	2.28	68.4	0.158	4.740	381	11,430
	Max.	4.5	5.06		0.956		810	
10	C	0.7	0.90	13.5	0.198	2.970	72	1,080
	15	1.7	1.36	20.4	0.222	3.330	198	2,970
	30	0.9	0.69	20.7	0.173	5.190	101	3,030
	Max.	3.4	3.50		0.860		504	
11	C	0.3	0.5	7.5	0.177	2.655	19.4	291
	15	4.4	2.98	44.7	0.294	4.410	159	2,385
	30	2.5	1.92	57.6	0.234	7.020	116	3,480
	Max.	7.3	13.2		2.083		521	
12	C	0.6	1.07	16.05	0.192	2.880	39.4	591
	15	3.7	4.28	64.2	0.740	11.100	288	4,320
	30	3.4	2.44	73.2	0.432	12.960	213	6,390
	Max.	5.0	3.00		0.516		276	
13	C	0.8	0.94	14.1	0.183	2.745	106	1,590
	15	4.3	5.36	80.4	0.434	6.510	950	14,270
	30	4.0	4.32	129.6	0.250	7.500	832	24,960
	Max.	6.0	9.40		0.223		1,210	
14	C	1.1	1.72	25.8	0.221	3.315	179	2,685
	15	3.3	5.10	76.5	0.374	4.610	623	9,345
	30	2.0	2.87	86.1	0.213	6.390	389	11,670
	Max.	6.0	8.58		0.782		1,410	
15	C	2.4	2.06	30.9	0.508	7.620	391	5,865
	15	7.0	6.59	98.8	0.716	10.740	1,575	24,625
	30	5.4	3.60	108.0	0.442	13.260	1,170	35,100
	Max.	8.6	10.98		0.488		2,228	
16	C	0.8	0.97	14.55	0.189	2.835	131	1,965
	15	4.7	7.35	110.2	0.236	3.540	783	11,745
	30	3.7	5.12	153.6	0.241	7.230	619	18,570
	Max.	5.3	8.24		0.247		862	
17	C	1.3	0.67	10.05	0.223	3.345	172	2,580
	15	2.1	1.32	19.80	0.257	3.855	321	4,815
	30	1.9	1.26	37.8	0.232	6.960	296	8,880
	Max.	3.0	1.43		0.475		489	

TABLE 6—*Concluded*

SUBJECT	PERIOD	CUBIC CENTIMETERS PER MINUTE	TRYPSIN		AMYLASE		LIPASE	
			Units per minute	Total	Units per minute	Total	Units per minute	Total
18	C	3.7	9.98	149.7	1.210	18.150	788	11,820
	15	13.8	14.4	216.0	2.256	33.840	1,963	29,445
	30	8.3	12.24	367.2	1.492	44.760	1,658	49,740
	Max.	18.0	38.4		7.191		4,460	
19	C	1.1	2.26	33.9	0.213	3.195	263	3,945
	15	10.4	17.6	264.0	1.057	15.855	1,915	28,725
	30	6.4	10.3	309.0	0.754	22.620	1,040	31,200
	Max.	20.0	36.2		1.141		3,600	
20	C	0.8	1.77	26.55	0.175	2.625	Lost	Lost
	15	5.2	12.15	182.3	0.752	11.280	1,081	16,215
	30	3.0	6.80	204.0	0.396	11.880	635	19,050
	Max.	9.3	24.36		2.502		2,105	
21	C	1.4	2.46	36.9	0.210	3.150	223	3,345
	15	6.0	12.90	193.5	0.576	8.640	1,640	24,600
	30	4.8	7.95	238.5	0.303	9.090	877	26,310
	Max.	14.0	21.36		1.315		1,925	
22	C	1.8	3.74	46.1	0.204	3.060	315	4,725
	15	7.5	14.56	218.4	0.603	9.045	1,049	15,735
	30	4.9	10.80	324.0	0.366	10.980	805	24,150
	Max.	18.4	32.22		1.205		2,110	

evacuation of the gall bladder had occurred. As may be seen from a consideration of the data (table 5), no increase in either of these two substances is seen which is significant of even a moderate gall bladder evacuation, after injection of secretin. While a small increase in the bilirubin values is seen in 10 of the 22 instances after secretin injection, this increase is too small and inconstant to have an important rôle in a consideration of the final results. These figures considered together with an absence of increased specific gravity and color change following the injection of secretin constitute evidence that no significant gall bladder evacuation had occurred.

Pancreatic enzymes. Our investigation of the response of the human pancreas to secretin correlates well with the experimental work on dogs recorded above. Thus, in the human we were unable to demonstrate any constant increase in the concentration of the enzymes in the duodenal drainage after an injection of secretin. From table 7 it may be seen that during the period of maximum flow in the 22 tests the concentration of

TABLE 7

Showing the concentration of enzymes in the duodenal drainage before and after the injection of secretin

Enzymes shown as units per cubic centimeter. Tests were made on the fluid obtained during the period of maximal flow of duodenal fluid.

SUBJECT	PERIOD	TRYPSIN	AMYLASE	LIPASE
1	Control	2.38	0.346	90.0
	Post-injection	1.90	0.090	60.0
2	Control	2.38	0.148	62.0
	Post-injection	1.86	0.122	86.0
3	Control	0.46	0.118	8.0
	Post-injection	0.86	0.0279	64.0
4	Control	2.38	0.765	248.0
	Post-injection	1.59	0.097	212.0
5	Control	1.37	0.328	102.0
	Post-injection	3.16	0.120	146.0
6	Control	1.07	0.114	54.4
	Post-injection	1.85	0.196	55.0
7	Control	0.515	0.216	Trace
	Post-injection	1.085	0.057	106.0
8	Control	0.25	0.088	32.4
	Post-injection	0.57	0.193	49.6
9	Control	1.14	0.410	92.4
	Post-injection	1.13	0.212	180.0
10	Control	1.28	0.283	101.0
	Post-injection	1.03	0.253	148.0
11	Control	1.65	0.590	64.8
	Post-injection	1.78	0.285	70.4
12	Control	1.80	0.320	65.6
	Post-injection	0.60	0.103	55.2
13	Control	1.27	0.229	198.7
	Post-injection	1.56	0.037	201.0
14	Control	1.58	0.201	162.4
	Post-injection	1.43	0.130	235.0
15	Control	0.86	0.212	163.0
	Post-injection	1.28	0.057	260.0

TABLE 7—*Concluded*

SUBJECT	PERIOD	TRYPSIN	AMYLASE	LIPASE
16	Control	1.22	0.249	163.2
	Post-injection	1.52	0.047	162.4
17	Control	0.515	0.172	132.4
	Post-injection	0.486	0.158	163.0
18	Control	2.70	0.327	213.0
	Post-injection	2.13	0.400	253.0
19	Control	2.06	0.206	239.0
	Post-injection	1.76	0.057	180.0
20	Control	2.22	0.219	Sample lost
	Post-injection	2.62	0.269	226.0
21	Control	1.75	0.150	159.2
	Post-injection	1.52	0.094	137.6
22	Control	2.08	0.113	174.0
	Post-injection	1.77	0.065	114.0

trypsin was decreased following secretin injection in 11 instances and increased in 10. Likewise the concentration of amylase was decreased after secretin in 17 out of the 22 tests. It should be noted, however, that a decreased concentration of trypsin did not always occur in the same subject in which a decreased concentration of amylase was seen. Lipase concentration was increased more uniformly being greater after secretin in 13 of 21 tests. In the total series of 22 tests the concentration of all of the enzymes was increased simultaneously in only 2 instances and all were decreased in 6 instances, although the increase or decrease in the concentrations of any one enzyme was never of the same magnitude as that seen in the other two in any test. In the remaining 14 subjects the concentration of the individual enzymes after secretin became greater or less in an apparently random fashion.

The total production or output of enzymes *per minute during the period of maximum flow* was increased after injection (table 6) except in one instance (subject 15, amylase; it is to be noted that in this case the control amylase was very high). In the case of trypsin this increased rate of enzyme secretion after secretin injection per minute during the period of maximum flow was from 2 to 160 fold (average 17-fold) as compared to trypsin secretion during the control period. The rate of amylase secretion following secretin was from 1.2 to 20.8-fold (average 5.8-fold) as during the control period. Total lipase production per minute during the period

of maximum flow was increased to from 3 to 700-fold (average 45-fold). We do not believe that the relatively enormous increases in output represent actually the minute output of the pancreas itself, but rather indicate that juice previously secreted was ejected rapidly through the tube by a contraction of the duodenum.

If the *average per minute production* of the individual enzymes for an *arbitrary period of 30 minutes* after injection is calculated (table 6), it is seen that this value is lower than the control period in only 7 instances. Since in the entire series the production of three enzymes was followed on each of 22 subjects (66 determinations of the individual enzymes), the 8 instances above represent in reality an incidence of slightly over 10 per cent in which the total production of enzymes over a 30-minute period was not greater than during the control period. Six of the 7 instances concerned the total production of amylase and one the total output of trypsin.

TABLE 8*

Showing the volume flow of fluid in cubic centimeters per minute before and after secretin injection

PERIODS	MAXIMUM	MINIMUM	AVERAGE
	cc. per minute	*cc. per minute*	*cc. per minute*
Control	3.7	0.2	1.13
15 minute period:			
The 20 normal subjects	13.8	3.3	5.5
The 22 subjects	13.8	1.7	5.3
30 minute period:			
The 20 normal subjects	8.3	2.0	4.1
The 22 subjects	8.3	0.9	3.9

However, the total output of lipase was increased in every instance. This must mean that, in view of the fact that the total production of each enzyme during the period of maximum flow is practically always increased, this period of maximum flow is followed in some normal subjects by a period of decreased activity of the pancreas in enzyme secretion. Analysis of the individual curves of the enzyme output in the successive samples of the various subjects shows this to be the case usually, the data being too bulky to publish.

Thus, if this period of decreased activity be included in a summary of the pancreatic response to secretin, it may, as shown above, decrease the total enzyme response in 10 per cent of instances to a value approaching or even falling short of the control secretion. We would therefore be inclined to

* During the period of maximum flow, the response per minute of the 20 "normals" was from 4.5 to 44 cc., averaging 11.8 cc.; that of the 22 subjects from 3.0 to 44 cc., averaging 10.6 cc.

limit the observation of pancreatic response to secretin in regard to minute output of enzymes to a fifteen minute period following the injection. If this period is arbitrarily adopted, it will be found to include the period of maximum flow and exclude most or all of the period of "depression" of enzymic secretion or the "washing out" of the enzymes which apparently follows secretin stimulation. This conclusion is borne out by the fact that if the shorter period (15 minutes) is substituted for the 30 minute period above, there is only one instance (amylase subject 7) in which the total production or output of enzymes for the post-injection sample is less than during the control. However, a study of known pathological cases may show the 30 minute period to be preferable. A calculation of the total production of enzymes by the pancreas implies a quantitative drainage of the duodenum by the Rehfuss tube, a condition certainly not accomplished with the present method of duodenal intubation. It is not known, of course, but that in the above seven instances at least a considerable amount of the duodenal contents may not have been delivered by the Rehfuss tube, although it would be expected that this error would be constant for all subjects. This same factor of incomplete drainage has not been of paramount importance in gastric analysis, although in this connection drainage of the stomach is probably more quantitative than is a similar procedure carried out on the duodenum. To our knowledge no one has determined what portion (or the individual variations) of the duodenal fluid is delivered by a duodenal tube.

In the human, as was noted in the dog, secretin apparently causes a greater and more constant secretion of lipase than of the other two enzymes. In the human, however, the production of trypsin is apparently more uniform than is the production of amylase. The fact that the production of amylase is the most easily exhausted of any of the enzymes may explain in part why certain individuals are troubled with flatulence after the ingestion of foods high in starch.

The *total output of enzymes* during the different periods is given in terms of units of enzyme activity per minute (average) for the 22 subjects in table 9.

Possible enzyme output deficiencies in these presumably normal subjects. It has been pointed out above that we believe subjects 10 and 17 are deficient in their response to secretin in so far as fluid output is concerned. In regard to lipase output, subjects 1, 3, 6, 8, 10, 11, 12 and 17 are low. Of these subjects, only 10 and 17 have digestive disturbances or free fat in the stools when a meal containing much fat is ingested. We could detect nothing of an abnormal nature in these subjects in regard to the amylase response or starch splitting deficiency. In regard to trypsin the output of subjects 10 and 17 is low, but on eating one-half pound of rare beef, meat fibers are absent from the stool.

General reactions to secretin. The intravenous injection of a standard dose of secretin (15 mgm. or 300 dog threshold doses) caused some flushing and warmth of the face in 19 of the 22 subjects with no other immediate objective or subjective symptoms. In general, the intensity of the flush regulated the speed of the injection. Usually half of the dose was given during the period of one minute. Depending upon the degree of the flush, the remainder of the dose was either given immediately or over a period of three minutes. About one-half of the subjects complained of headache some time after the conclusion of the test, i.e., after the tube was removed. Whether this was secondary to the secretin injection or the duodenal intubation alone is problematical. In one instance a generalized patchy urticarial eruption occurred while the subject was taking a hot bath three hours after the injection. This subsided in 1½ hours. In the two subjects

TABLE 9

Average total output of enzymes by the pancreas in 22 human subjects

Expressed in units of enzyme activity per minute

	AMYLASE			LIPASE			TRYPSIN		
	Minimum	Maximum	Average	Minimum	Maximum	Average	Minimum	Maximum	Average
Control*	0.059	1.210	0.375	Trace	788.0	152.7	0.24	9.98	1.72
15 minutes after injection† *	0.211	2.256	0.526	159	1,963	762	1.32	17.60	7.12
30 minutes after injection†	0.158	1.492	0.327	101	1,658	533	0.69	12.24	4.57

* Total output of enzymes in units for the period may be obtained by multiplying by 15.

† Total output of enzymes in units for the period may be obtained by multiplying by 30.

who submitted to a second injection of secretin after an interval of 17 months, however, a severe generalized reaction occurred. (This secretin preparation had produced no reaction in 12 subjects who received it as an initial injection.) Both subjects felt well after the experiment and consumed a little food. Shortly after and *one hour* after the secretin injection, a headache was experienced, and then about 20 minutes later a severe shaking chill accompanied by abdominal cramps, nausea and vomiting resulted. Dull aching joint pain and low lumbar pain was complained of. Diarrhea and polyuria were present in one subject, while in the other an anuria of 14 hours duration, and constipation was observed. On the 3rd day (60 hours) following the injection, a herpetic eruption involving the lips and buccal mucosa developed in both subjects. This eruption was more severe in the subject who previously exhibited anuria and constipation, eventually

spreading to involve the upper lip and chin. This eruption disappeared in the course of a week. The passive transfer test was negative and the intracutaneous injection of 0.2 mgm. of secretin gave a result which could not be interpreted as a positive reaction. We had previously shown that this preparation of secretin was non-antigenic in the guinea pig and dog, that is, neither precipitins nor anaphylaxis was produced. In view of this and the negative intracutaneous and passive transfer tests, these instances of acquired sensitivity to the secretin preparation must have been some peculiar allergic manifestation. Subsequently the intracutaneous injection of 0.2 mgm. of this secretin gave identical reactions in not only all of those who had previously received a secretin injection but also in a number of individuals who had never received secretin. This reaction consists of a moderate flare of considerable size and slight swelling of the injected site and is apparently of a non-specific nature. By blood pressure assay 5 mgm. of the dry powder is free from vasodilatin in dogs weighing 15 kilos. It is to be recalled that this secretin preparation contains no aniline and its nitrogen content is only 6.9 per cent.

Repeated trials on the same subject. A consideration of the data shown in table 10 shows that while the curves obtained on repeated tests on the same individual are similar they are not comparable in a sense that allows an absolute comparison. The values for specific gravity, bilirubin and cholesterol obtained show that there was no evacuation of the gall bladder in any of the four tests. The divergent results cannot therefore be explained on this basis. These facts, together with a consideration of the fluctuating character of the enzyme concentration seen in the other human tests and in the dog, strengthens our belief that the enzymic concentration alone found before or after secretin injection is of little or no value as a criterion of pancreatic function. Calculation of the total output of enzymes before and after secretin injection shows, as in the dog, that an increase always occurs during the 15 minute period after the injection. However, the individual enzymes are not always increased to the same degree in the same test or in different tests on the same individual, using a standard dose of secretin.

It should be pointed out that the data recorded in the tables were compiled from 1031 chemical analyses, over two-thirds of which were performed on the duodenal drainage from the human series. This fact is mentioned to indicate that many more analyses were made than are practical to include in the tables which have been compiled in such a manner as to clarify and simplify presentation.

Discussion. The experimental work that we have performed on both canine and human subjects does not allow us to agree fully with the statement of Chiray and his co-workers that, following secretin injection, the concentration of pancreatic enzymes in the duodenal drainage is in-

creased from two to threefold. On the basis of what is known of the characteristics of pancreatic juice formed in anesthetized animals in response to secretin injections, the results of the forementioned authors are difficult to explain, for it has been shown (10) that this type of pancreatic juice is poor in enzymes as compared to the "vagus secretion." However,

TABLE 10

Comparison of results in two different tests on the same patient

PERIOD	CUBIC CENTIMETERS PER MINUTE	TRYPSIN		AMYLASE		LIPASE	
		Units per minute	Total	Units per minute	Total	Units per minute	Total
			Subject 2				
			First run				
Control	1.2	2.76	41.4	0.178	2.670	75	1,125
15 minutes	5.2	9.24	138.6	0.466	6.990	464	6,960
30 minutes	2.9	6.78	203.4	0.261	7.830	397	11,910
Maximum	7.7	14.80		0.941		662	
			Second run				
Control	5.7	9.35	28.5	1.030	15.450	550	8,250
15 minutes	11.7	15.6	234.0	2.135	32.025	1,762	26,430
30 minutes	8.5	11.5	345.0	1.456	43.680	1,135	33,950
Maximum	13.8	17.4		2.524			
			Subject 3				
			First run				
Control	0.5	0.24	3.6	0.059	0.885	4	60
15 minutes	4.9	3.43	51.5	0.215	3.225	238	3,570
30 minutes	3.4	2.40	72.0	0.192	5.760	163	4,890
Maximum	44.0	38.2		1.225		2,830	
			Second run				
Control	0.7	Trace	Trace	Trace	Trace	Inactive	Inactive
15 minutes	4.0	2.70	40.50	0.721	10.810	528	7,928
30 minutes	3.4	1.95	58.50	0.538	16.150	439	13,170
Maximum	4.7	3.57		0.827		531	

in a series of 5 unanesthetized dogs with chronic pancreatic fistulae in this laboratory, we have shown (vide supra) that the enzyme concentration of "secretin pancreatic juice" may be increased over that of a fasting control sample in about 40 per cent of the cases. In this connection it is interesting to note that a similar discrepancy is to be found in the literature dealing with the stimulation of the glands of the stomach with histamine. Some

authors report that the injection of histamine in the dog increases the total output of pepsin in the gastric juice, while others claim that the pepsin concentration and total output are routinely lowered (10). In man (11) according to some authors, an injection of histamine raises the concentration and total output of pepsin in the gastric juice; according to others, the concentration is decreased and the total output increased (12). It is possible that these variable results may be explained on a basis of dosage; relatively small doses per kilo such as are given in man cause the production of a comparably small amount of juice relatively rich in enzymes, while the injection of larger doses, as in the dog, cause the secretion of an abundant gastric juice poor in enzymes. Since the potency of the secretin used by Chiray cannot be compared to the material used by us, it is impossible to say whether or not such a mechanism might explain the discrepancy in our results as well. To our knowledge, the question of secretin dosage in relation to the enzyme concentration of pancreatic juice has not been investigated.

We have presented evidence to show that no significant evacuation of the gall bladder occurred in our series following secretin injection; the lower concentration of enzymes in our post-injection samples cannot be due to dilution with gall-bladder contents. On the other hand, Chiray and his co-workers present no analysis for magnesium in their pre-injection control samples to show that these samples are not considerably diluted with magnesium sulphate solution, hepatic bile or succus entericus remaining after the flushing of the duodenum with the former substance. The use of C bile instead of the normal contents of the fasting duodenum as control samples by these authors would in itself tend to lower appreciably the concentration of pancreatic ferments in their control samples. It is further possible but not so likely, that these authors used a preparation of secretin that stimulated the duodenal glands as well as the pancreas, resulting in a fortification of the pancreatic enzymes with intestinal ferments, especially lipase. The secretin preparation used by us does not appreciably stimulate the duodenal glands as we have shown by experiments on Thiry fistula dogs.

An interesting observation is the marked variability with which the individual enzymes respond to a standard dose of secretin. It is impossible to predict, either on different tests on the same individual or on tests on different individuals, whether a given enzyme will be increased or decreased in its concentration or whether its total production will be only slightly or markedly increased. This shows the impracticability and fallability of basing conclusions as to pancreatic function on a consideration of the enzyme concentration alone and particularly when the duodenal drainage is assayed for only one enzyme. Whether or not the concentration of an

enzyme will rise following secretin injection depends to a large measure on the concentration found in the fasting control sample. Since this latter value might be normally very high, the entire consideration of enzyme concentration becomes an entirely relative matter.

The total production of enzymes over a post-injection period of only 15 minutes, however, showed only one instance in which the total output of enzymes was less than during an equal control period. We have therefore designated the latter time as the optimum period over which to observe the pancreatic response to secretin.

Since the increased production of enzymes is so frequently manifested by an increased flow of a fluid containing a decreased concentration of enzymes, the minute volume output of fluid and enzymes is a factor of prime importance in interpreting the pancreatic response to secretin. In this regard we have ascertained that the subjects showing the poorest response to secretin (subjects 10 and 17) manifest steatorrhea at least occasionally. A patient showing a response not exceeding the one obtained in these subjects may be suspected of having at least potential pancreatic insufficiency, although we do not believe that at the present time a positive diagnosis could be made from a single secretin test.

In obtaining the response of the pancreas to the injection of a standard dose of secretin we advocate the following procedure:

1. Pass the duodenal tube and check its position by fluoroscopy. The olive should rest in the second portion of the duodenum.

2. Collect drainage samples from the duodenum noting the time carefully. For enzyme analysis reject all samples that are turbid or acid in reaction. When the control drainage has fallen to a "basal" rate, begin collection of control samples. The foregoing is of great importance as otherwise a control sample composed of the concentrated fasting output of pancreatic juice may be obtained at an apparently rapid rate of production. This basal flow exceeds 2 cc. per minute only occasionally.

3. Following the collection of one or more suitable control samples covering a period of 15 minutes, an intravenous injection of 300 dog threshold doses of secretin (15 mgm.) is given. The response of the pancreas should be followed over a 15 and 30 minute period. We would advise the collection of the 15 minute quantity in a number of individual samples so that the rate of maximum minute volume output may be determined.

4. Analysis for enzymes should include the control, the period of maximum flow and the combined post-injection samples excluding the portion covering the maximum flow. The total output for a 15 and 30 minute period may then be calculated from these data.

5. Samples should be placed in the ice-box upon collection and should be analyzed for enzyme content immediately following the test as these enzymes, especially amylase, may deteriorate rapidly after removal from the duodenum, if permitted to remain at room temperature.

In interpreting the results of the test we believe it misleading to consider enzyme concentration in the samples unless, of course, the concentration is abnormally low or one or more of the enzymes is absent. A calculation of the total output of enzymes in normal subjects should always show a greater output of all enzymes after secretin injection when compared to the control. This increased production should be evident not only in the period of maximum flow but also, to a less marked degree, for the 15 minute post-injection period. Arbitrary limits for the production of enzymes following secretin injection cannot be fixed because of the variability of enzyme concentration in the control flow and in the minute volume of control drainage. In general it may be said that the rate of enzyme production in the normal individual should be above the minimum shown in table 9 following secretin injection. The flow of fluid should be tripled or equal to the minimum figures shown in table 8. Individuals falling below the minimum may be suspected of having pancreatic disease, although the exact criteria for a diagnosis of this sort can only be made after the study of a large number of pathological cases.

This paper has been designed for the purpose of ascertaining the normal response of the pancreas of man and dog to standard doses of secretin. This work is intended to precede and form the foundation for the development of an adequate pancreatic function test utilizing secretin as the stimulating agent. The realization of such a pancreatic function test depends on: 1. The investigation of a number of pathological cases in respect to their response to standard doses of secretin; 2, the preparation of crystalline secretin, or at least a purified product that is innocuous upon repeated injections into humans; 3, the standardization of a procedure which will give comparable results in the hands of various investigators.

It is believed that in pathological cases the secretin test should be accompanied by an examination of the stools in order to ascertain the relative merits of the two examinations. It will probably be found that the secretin test has the same relative merits in testing pancreatic function that histamine has in testing gastric secretory function. It should be obvious that the results of a secretin test will not necessarily be a test of intestinal digestion regardless of the fact that pancreatic secretion plays such an important rôle in intestinal digestion, motility and the quantity and quality of intestinal juice and bile being other important factors.

SUMMARY AND CONCLUSIONS

1. In the dog and in man there is no consistent increase in the concentration of pancreatic enzymes found in the duodenal drainage after the intravenous injection of secretin. In tests made on 22 human subjects a simultaneous rise in the concentration of amylase, lipase and trypsin in the duodenal contents after secretin injection was noted in only 1 instance.

In 6 instances, the concentrations of these enzymes were simultaneously decreased. In the remaining 15 tests the concentrations of these enzymes were increased or decreased in an apparently random fashion.

2. The total output of all the enzymes (trypsin, amylase, lipase) is almost invariably increased during the first 15 minute period after secretin injection in both the dog and in man. With the doses of secretin used, the fluid output should be nearly tripled during the 15 minute post-injection period. The results of secretin stimulation must be interpreted from an increased total production of enzymes and fluid. This fact is unfortunate in that it requires for absolute quantitative conditions, at least, a quantitative drainage of the duodenum, a condition probably not attained by the present methods of duodenal intubation. The average, maximum and minimum values for the production of enzymes and fluid before and after secretin injection are given in the text.

3. Repetition of the test on two subjects resulted in stimulation of the pancreas in both instances but did not result in strictly constant results. In other words, the response of the same individual to secretin varies from time to time.

4. A tentative procedure for testing the pancreatic response to secretin together with our interpretation of the results obtained has been given.

5. In view of the severe reaction that occurred in two subjects on receiving a second injection more than one year after the first injection, we believe that this preparation of secretin should be injected only once.

6. While no pathological cases were purposefully included in this work, we believe that the test has pointed out two cases of pancreatic deficiency in subjects that were presumed to be normal. However, we believe that it has yet to be established that any test based on duodenal drainage is of more practical value than stool analysis. This statement is made to encourage, rather than to discourage, further investigation in this field.

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SUPERNORMALITY, A MODIFICATION OF THE RECOVERY PROCESS IN NERVE

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Adrian and Lucas (1912) established the fact that the relatively refractory period of isolated frog nerve is followed by a period of supernormality during which the irritability of the nerve is greater than before stimulation, and Adrian later (1920) showed that the supernormality varies with the hydrogen ion concentration of the fluid bathing the nerve, increasing both in magnitude and duration as the hydrogen ion concentration increases. Gasser and Erlanger (1930) pointed out that the supernormal period varies with the after-potential, and that in harmony with this relationship it may be absent in fresh nerves. They also observed that supernormality is purely a phenomenon of thresholds, the maximum height of response never rising above its resting level. This height they showed to be reached in normal nerve in the interval after a response required for the irritability to reach its resting value, i.e., at the start of the supernormal period when this exists.

It is evident from Adrian's figures that the relatively refractory period as ordinarily conceived in terms of irritability (the interval after a response at which the nerve becomes as irritable as before) is shortened when supernormality develops under the influence of increased hydrogen ion concentration; at the same time the level of resting irritability is depressed, so that the maximum (supernormal) irritability of the "acid nerve" is no greater than the resting irritability of the "neutral nerve." The phenomenon of supernormality may therefore be merely the expression of the inability of the nerve to maintain its normal resting irritability, which it can nevertheless temporarily reach in the usual interval after a response. This interpretation implies that the recovery process proper has not been modified by the change in hydrogen ion concentration; and as a matter of fact, when allowance is made for the depression of resting irritability, the recovery curves at neutral and at acid pH are superimposed up to about 75 per cent of the normal (neutral) irritability level and differ but little in their remaining course to maximum irritability.

The view adopted by Adrian is that the recovery process itself is not

modified by the development of supernormality in acid nerve, but is merely followed by a fall of irritability not occurring in neutral nerve. This view rests upon the assumption that the maximum supernormal irritability does not exceed the level of resting irritability of the same nerve under "normal" conditions. The greater the depression of this level, the greater the supernormality that may be developed, in the absence of any factors interfering with this development. Such a reciprocal relationship between level of resting irritability and degree of supernormality is likewise suggested by the effects of excess univalent and bivalent cations on these two functions of nerve (10). The following two published facts, however, militate against this view: 1. Adrian (1920) reports that alkalinity decreases supernormality while also depressing resting irritability. This apparent exception (or any other similar case) may however be explained away by the assumption of some secondary interference with the manifestation of supernormality. 2. Veratrine (12) has been reported to increase resting irritability and to prolong supernormality. If the observations are correct, and unless the prolongation is accompanied by a diminution in degree of supernormality, this represents a valid argument against the conception of supernormality as merely a transient normal resting irritability, and indicates that the development of supernormality represents a modification of the recovery process.

Experiments designed to find out which of the two alternative interpretations of supernormality is correct are presented in this paper. Since the increased capacity to exhibit supernormality induced by excess hydrogen ion or bivalent cation concentration has already been studied in its relation to the accompanying changes of irritability and of recovery of irritability, the present experiments were confined to the changes occurring spontaneously in isolated nerve and to the effects of veratrinization.

METHOD. The nerve used in most of the experiments was the isolated sciatic of the green frog (Rana pipiens) which was mounted in a moist chamber on stimulating and recording electrodes, and was rendered relatively monophasic by heating at or near the grid lead. In measurements of the conduction rate where long conduction distances were required, the peroneal or posterior tibial branch was included in the preparation; still greater length was secured by using bull frog nerves (Rana catesbiana) in a few experiments. In order to veratrinize a green frog nerve when desired, a neutralized veratrine hydrochloride solution (1/50,000 to 1/250,000) was painted on it without changing its position on the electrodes. The bull frog nerves were not easily veratrinized by this method and therefore when veratrinization was desired, were immersed in the veratrine solution for $\frac{1}{2}$ to 1 hour before mounting.

The cathode ray oscillograph used was the instrument described in earlier publications from this laboratory (10). The stimuli were induction

shocks produced either by breaking the primary circuit of a Porter inductorium, or by the Thyratron stimulator of Schmitt and Schmitt (14). In most of the experiments it was necessary to have two shocks (the conditioning shock and the testing shock) independent of each other in time and in strength. For ease of reproducibility and constancy of shape of shock, Thyratron stimulation with variable resistance in parallel with the nerve was used as far as possible. Variation of such a shunt resistance however affects the strength of both shocks when they are applied at the same pair of electrodes. The testing shock in irritability measurements was nevertheless usually regulated in this way, while in order to minimize mutual interference, the conditioning shock, when applied at the same electrodes, was controlled by changes of the primary resistance of the inductorium when this was used, or by changes of the resistance or capacity in the discharge circuit when the Thyratron stimulator was used for this shock. The testing shock was thus entirely independent of variations in the strength of the conditioning shock, while the changes induced in the conditioning shock by variations of the testing shock could be rendered ineffective either by the use of a conditioning shock strong enough to produce a maximal response for all values of the shunt resistance used, or by varying the resistance or capacity in the conditioning shock circuit. Such variations of course affected the shape as well as the strength of the conditioning shock when this came from the Thyratron stimulator, but this theoretical objection proved not to make any difference in the results.

A variation of 1 to 5 ohms (0.3–1 per cent) in the shunt resistance was usually the accuracy with which the irritability to the testing shock was measured. The strength of the stimulus was proportional to the variable resistance within an amount determined by the relation of this resistance to the resistance of the nerve and the fixed resistance in the secondary circuit (5000–10,000 ohms used); under the conditions (10,000–30,000 ohms resistance of the nerve, and 100–200 ohms in the decade resistance box for threshold stimulation), the error involved in assuming the strength of stimulus to be proportional to the variable resistance is less than 1 per cent for a 10 per cent change of strength of stimulus, and less than 3 per cent for a shock of twice threshold strength. Since the error is thus relatively small, this assumption was made in calculating and plotting the changes of irritability; correction of the error would not materially affect the course of the irritability curves as plotted.

The changes of irritability after a conditioning response were followed by varying the strength of the testing shock so that it evoked a fixed small (threshold or low submaximal) amplitude of response (5). The conditioning shock was made at least strong enough to stimulate all fibres capable of responding to the testing shock at maximal supernormality. The height of the conditioning response was never less than twice that of the uncon-

ditioned testing response and was sometimes maximal. The method of measuring irritability involves an error in observations made during the refractory period, inasmuch as the decreased height of response during this period necessitates the stimulation of more fibres in order to produce the fixed amplitude of response. A greater number of fibres and consequently less irritable fibres must be stimulated as the interval between the conditioning and testing shocks is shortened. This and the fact that less irritable fibres have longer refractory periods (3) will make the observed value of irritability at any refractory interval somewhat less than the actual value. The discrepancy will become smaller as the normal height of response is approached with increasing length of interval between the shocks and will disappear entirely at the beginning of the supernormal period. Measurements of the length of the relatively refractory period will therefore not be affected at all by this error, and as the error is presumably of similar extent in the various conditions studied, the various recovery curves may be regarded as comparable.

Recovery of height of response was generally measured by selecting a low submaximal height of conditioning response, and a height of testing response not less than twice as high (8); maximal responses (A fibres only) were sometimes observed in addition. When the recovery of height and that of irritability were to be observed on the same preparation, the height of one response was made approximately one-half that of the other, both being submaximal; in order to ensure observations being made on the same fibres for the two curves without time-consuming readjustments of shock strength, the position of the keys on the rotator was shifted so that the rôle of the two shocks was reversed in the two series of observations, the testing shock in the irritability measurements becoming the conditioning shock in the height series and vice versa.

The rate of conduction was determined by direct observation on the screen of the time between the start of the shock artifact and the start of the nerve action potential after conduction over distances ranging from 3 to 20 cm.; or by measuring this time after stimulation at two different points and by subtraction determining the time of conduction between them. Changes in rate late in the refractory period and throughout the supernormal period are too slight to be measured accurately if the whole interval required for conduction is thrown upon the screen of the oscillograph; on the logarithmic time line, a variation of about 5 per cent in the interval represents the change that can be read with any degree of ease and accuracy. For slight changes in conduction time, therefore, the normal conduction time was observed as usual; then the oscillograph was adjusted to record the testing response on a very rapid time line, and the shift in position of the response observed when the conditioning shock was introduced. Changes amounting to small fractions of a sigma are thus easily

observable. Similarly arranged observations—always negative—of any change in position of the testing shock in consequence of the introduction of the conditioning shock, were made as controls.

The conditioning and testing shocks were applied in some experiments at the same electrodes, in others at two different pairs of electrodes, 1 to 2 cm. apart, with no significant difference in the results obtained.

RESULTS. I. *Spontaneous changes in isolated nerve.* a. *Irritability.* Immediately after isolation of a green frog sciatic nerve, it was dipped in Ringer's solution and mounted in the moist chamber. Its level of resting irritability was then determined with as little stimulation as possible, and the determination repeated at intervals throughout the experiment. While the irritability of some nerves was found to vary somewhat during the time ($\frac{1}{2}$–$1\frac{1}{2}$ hour) of increasing capacity to manifest supernormality, the variations were not large, were not always in the same direction, and could be reproduced by moistening the nerve, letting it dry out slightly, or by readjusting it on the electrodes. The variations were undoubtedly due to uncontrolled experimental factors, and were frequently absent, as in the case of the experiment plotted in figure 1.

b. *Relatively refractory period* (rise of irritability to its resting value). The recovery of irritability after a conditioning response was found to require 7 to 10σ in freshly isolated nerves (25°–29°); the irritability approached its resting value asymptotically, and did not rise above this value. In the course of $\frac{1}{2}$ to $1\frac{1}{2}$ hour as the capacity to manifest supernormal irritability developed, the interval required for the irritability to return to its resting value became shorter and shorter and eventually remained fixed at 1.5 to 2.5σ, where the curve crossed the resting level at a considerable angle. Four successive lengths of the relatively refractory period are plotted in figure 1. The initial period of 8.5σ was shortened to 5.2σ during observations of the degree of refractoriness at various intervals; this was further shortened to 2.9σ during observations on the newly acquired supernormal period; and a final value of 2.5σ was obtained somewhat later after other experimentation. Another illustration of the same change is found in the upper part of figure 2.

The curve of recovery of irritability is affected down to about 75 per cent of the normal level; below this point it has not been possible to demonstrate any significant difference before and after the capacity to exhibit supernormality has been developed. The greater the degree of refractoriness, the less the interval corresponding to it is shortened relatively as well as absolutely, and the absolutely refractory period and the curve of recovery up to at least 50 per cent of the resting irritability apparently remain constant even when the relatively refractory period is shortened to as little as one-quarter of its original value. If any shortening occurs in the early

part of the recovery curve it is insignificant enough to fall within the limits of experimental error.

c. *Supernormal period.* The rise of irritability above the resting value which occurs during the early part of the supernormal period is perfectly continuous with the rise during the refractory period, the steepness of the whole curve gradually decreasing with increasing interval after the con-

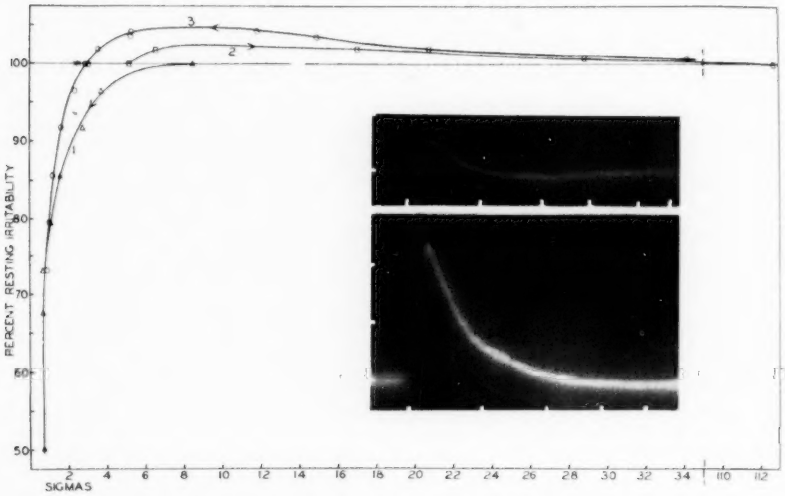

Fig. 1. Shortening of the relatively refractory period with the development of the supernormal period. Green frog sciatic nerve mounted in moist chamber promptly after isolation and subsequently untreated except by shocks required for observation. Conduction distance = 7 mm. for testing shock, height determinations and after-potential records; 24 mm. for conditioning shock. Points determined in order indicated on curves and by numbering of curves. × = observations at end of experiment after recording after-potential, maximal height of response and conduction rate. Resting irritability and maximal height of response constant throughout. Experiment required about an hour. Abscissae = interval between conditioning and testing shocks. Inset: After-potential records ($\times\frac{3}{4}$) before (upper) and after irritability observations plotted in curves 1–3. Abscissae = time in 25σ intervals; ordinates = potential in 0.1 mv. steps. Corresponding spike = 10.1 mv Temp. = 26°C. 9/29/33.

ditioning shock. The interval required for the attainment of maximum irritability can not be measured exactly either in the presence or in the absence of the supernormal period, but it can be shown by plotting the experimental data as in figure 1, that this interval remains the same for a given nerve in the two conditions. The maximum degree of irritability attained after the capacity to develop supernormality has developed, is

usually 105 to 110 per cent of the resting value, and has never been observed to exceed 120 per cent. The irritability remains at or practically at this maximum level for a number of sigmas which it is difficult to determine accurately, since the subsequent decline of irritability is even more gradual than the latter part of the rise; the period of declining irritability lasts many times as long as the period of rising irritability, and has already been shown to coincide in a general way with the decline of the after-potential (8).

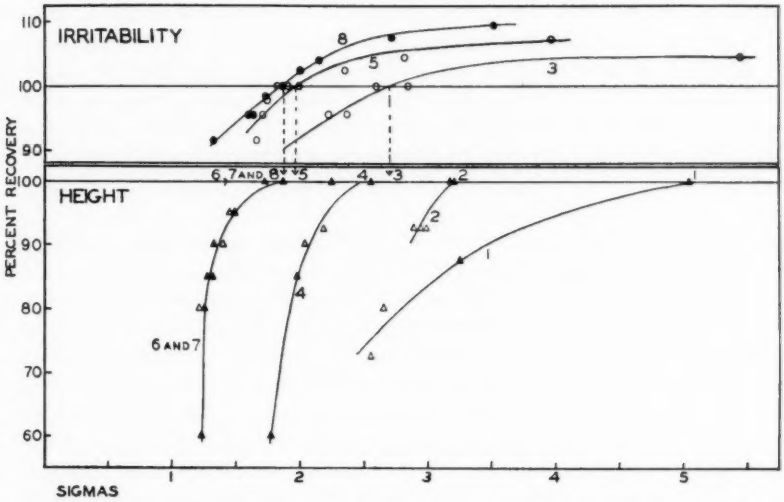

Fig. 2. Simultaneous acceleration of recovery of height and of irritability with development of the supernormal period. Green frog sciatic nerve mounted in moist chamber promptly after isolation. Conduction distance 14.5 mm. Curves 1 to 6 obtained in numbered order in course of $1\frac{3}{4}$ hour continuous observation (△ and ○). Nerve then stimulated maximally at rate of 15/min. for 20 min.; after 30 minutes' rest, curves 7 and 8 obtained (▲ and ●). Abscissae = interval between conditioning and testing shocks. Temp. = 27.5°C. 11/1/33.

The above description of the development of supernormality applies in general to most of the nerves examined, but for unknown reasons supernormality did not develop at all in occasional cases. For reasons that are obvious from Adrian's work (1), the development is slower in nerves that have been bathed in alkaline Ringer's solution.

d. *Recovery of height of response.* Complete recovery of height is known to require the same interval as recovery of irritability in nerves with the capacity to exhibit supernormality (8). The progressive shortening of the relatively refractory period accompanying the development of this

capacity raises the question: is the interval required for recovery of height similarly longer in freshly isolated nerve, or is it fixed at the length to which the relatively refractory period ultimately shortens? Figure 2 presents the results of one of the experiments performed to answer this question. In this experiment, the interval required for recovery of normal height was first determined (5.1σ), and heights at three other progressively shorter intervals were then measured (curve 1). Slight lengthening of the interval then revealed more rapid recovery of height than before, and complete recovery at 3.4σ (curve 2). A portion of the recovery of irritability curve was then plotted, and was found to cross the normal irritability level at 2.7σ (curve 3). Immediate subsequent measurement of the recovery of height of response showed that normal height was now reached at 2.4σ, and ultimately both normal irritability and normal height of response were regained 1.88σ after the conditioning shock. The obvious conclusion from such results is that recovery of irritability and recovery of height vary together, and that at any given moment, the interval required for recovery of 100 per cent irritability or height is the same.

The normal height of response ordinarily does not change when the nerve acquires the ability to manifest supernormality.

e. *Conduction rate.* The rate of conduction has not been observed to change significantly as the power to exhibit supernormality develops. The important feature of conduction rate for the problem under investigation, however, is the possibility of its being increased during the supernormal period. It was shown by Gasser and Erlanger (7) that the delay of a testing response introduced into the relatively refractory period following a conditioning response is due to a slower rate of conduction, and that this slowing is decreased as the interval between the two responses is increased. Inasmuch as theoretical considerations indicate that the rate of conduction is dependent upon the irritability of the tissue and the amplitude of response, it seemed possible that the rate of conduction might be above normal during the period of supernormal irritability.

Because of failure to appreciate how limited the increase in rate during the supernormal period might be expected to be, it was overlooked in the first cathode ray oscillograph experiments (11); similar negative results were reported by Cooper, using an entirely different method. When observations during the latter part of the refractory period revealed the greatest magnitude of change in rate that could be expected in the supernormal period, a new series of experiments was performed, with longer conduction distances and provision for observation of smaller differences in conduction time. The longer conduction distances increased the absolute difference between the conduction time of the unconditioned response and that of the conditioned response, thus decreasing the influence of errors of measurement and the possible interference from variable latency (3). Spontane-

ous variations in the position of the testing response, presumably due to the latter factor, were frequently observed, but when they were of the order of the effects of introduction of the conditioning response, allowance for them could be made by averaging a number of observations of the position of the testing response first alone and then when preceded by the conditioning response.

Observations were made both on unpoisoned nerves showing considerable supernormality of irritability, and on nerves veratrinized for the purpose of increasing this supernormality. As the results obtained in the two series were qualitatively and quantitatively very similar, they will be treated as a unit. Of the 16 experiments with conduction distances of 60 mm. or over, all but two showed definitely supernormal conduction rates at certain intervals between the shocks; data from 8 of these are included in table 1. Of the remaining 6 positive experiments, two showed definite supernormality of conduction rate of too small degree to be worth quantitative measurement, while the other four exhibited supernormality of rate of the same magnitude as those included in the table, but are omitted because of incompleteness of the recorded data. The data for the nerve exhibiting the greatest degree of supernormality of irritability and of conduction rate have been plotted in full in figure 3; very similar curves were obtained for the eight nerves included in the table.

The supernormal conduction rates have been tabulated and plotted in terms of per cent of the normal rate, and the subnormal rates late in the refractory period have been treated in the same way in the figure. Since the intervals plotted are those between the conditioning and testing shocks, they differ from the intervals between the responses when these reached the recording electrode except when the conduction rate of the testing response is normal (identical with the relatively refractory period, fig. 3). The difference is insignificant throughout most of the supernormal period because of the long interval between the responses and the slow rate of change of conduction velocity with difference in interval. At the very beginning of the supernormal period, however, when the supernormal conduction rate of the testing response brings it into less and less supernormal regions as it proceeds down the nerve, the rate calculated from the observed conduction time will be somewhat less than the rate obtaining at the interval between the shocks, and somewhat greater than the rate obtaining at the final interval between the responses. This is the reverse of the situation during the refractory period, when the "observed" rate will be greater than the rate obtaining at the shock interval, and less than that obtaining at the final interval between the responses. The theoretically correct curve relating rate and shock interval would cross the normal conduction rate level at the same interval as the one plotted, but would have a steeper slope; it would thus more nearly parallel the irritability

TABLE 1

Degree of supernormality of irritability and of conduction rate

	CONDUCTION DISTANCE	CONDUCTION RATE	MAXIMUM SUPERNORMALITY (PER CENT NORMAL)		TEMPERATURE	DATE
			Irritability	Conduction rate		
A. Green frog sciatic						
	mm.	*mm./σ*			*°C.*	
Unpoisoned	81	50.0	109.6	105.3	22.5	11/10/33
Unpoisoned	120*	36.9	105.4	110.3	21.5–22.5	11/13/33II
Veratrinized	60	44.1	113.2	110.6	25.5	10/16/33
Veratrinized**	80	45.5	119.2	113.5	24.0	10/19/33
B. Bull frog sciatic						
Unpoisoned	180–11	37.5	109.6	105.6	22.5	3/ 2/34
Unpoisoned	130–14	22.5‡	105.2	105.8	21.5	2/22/34II
Veratrinized	219–16.5	29.3	108.7	105.0	19.5–20.5	2/28/34II
Veratrinized	188–36	24.7	110.5	109.3	22.5	2/21/34I

* Conduction distance for conditioning shock, 130 mm.

** Plotted in full in figure 3.

‡ The fact that this nerve was mounted in such a way as to be stretched by its own weight presumably explains this slow conduction rate; its irritability was also subnormal.

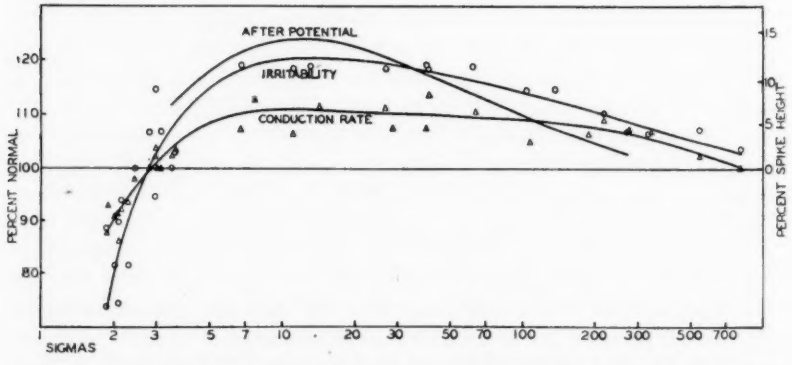

Fig. 3. Conduction rate during late refractory period and supernormal period. Green frog sciatic nerve, 1 to 3½ hours after application of 1/50,000 veratrine hydrochloride. Conduction distance 80 mm. Unconditioned conduction rate 45.5 min./σ. Ordinates: left hand scale, per cent of unconditioned irritability and conduction rate; right hand scale, per cent of spike height for evaluation of after-potential. Abscissae = interval after conditioning shock on logarithmic time scale. Temp. = 24°C. 10/10/33.

curve. The error involved in the method of plotting used would shift the curves in the experiment plotted less than 0.2σ when the conduction rate varies from the normal by 10 per cent. This is obviously of no importance at a rate of 110 per cent, where the interval is about 8σ; and the shift decreases as the supernormality decreases, so remains unimportant throughout the supernormal period. When the refractory period is entered, the shift starts increasing again, of course in the direction opposite to that during the supernormal period. With increasing refractoriness as the interval is shortened, the correction eventually becomes so great that a curve plotted as in figure 3, in terms of percentage of normal rate, becomes misleading. In these experiments, however, the observations were restricted to the portion of the refractory period where the curve retains some exactness, even though as may be seen in the figure, a shift of 0.2σ at 90 per cent would noticeably alter the course of the curve.

These observations were so carried out that a number of fibres were involved in the testing response chosen: and the different refractory periods of different fibres (3) might affect the result, since the most irritable fibres might already be supernormal at intervals short enough to leave less irritable fibres still refractory. At intervals close to the relatively refractory period, especially in badly deteriorated nerves, introduction of a conditioning response occasionally split the testing response into two parts, one of which fell earlier and the other later than the unconditioned testing response; the faster fibres were followed in these cases, and no attempt was made to keep the height of the testing response up to any fixed level.

Figure 3 demonstrates not only that the conduction rate becomes normal at the end of the relatively refractory period, but also that its subsequent rise continues for about the same interval as the rise of irritability. Usually, as in the experiment plotted in this figure, the maximal supernormality of conduction rate is less than that of irritability when each is expressed in terms of percentage of its normal or unconditioned value. In this particular experiment, irritability at its maximum was 119.2 per cent of its unconditioned value, while conduction rate, although the highest observed value was 113.5 per cent (table 1), actually probably rose only to about 110 per cent (fig. 3) of its unconditioned value. In the other experiments in the table, the highest observed value of conduction rate was probably also somewhat too high, due to unavoidable observational error, so that in all cases the maximum percentage value of irritability undoubtedly exceeded the maximum percentage value of conduction rate by an amount larger than the table indicates.

The decreased conduction time in the supernormal period might conceivably be due to a local effect (such as a change of the latent period) at the electrodes, although the fact that decreased conduction times were

not found in the experiments with short conduction distances where the brief conduction times would accentuate any change other than in conduction rate, is against this possibility. The possibility was, however, tested further by applying the conditioning and testing shocks at different points on the nerve, as in the second experiment recorded in the table; the decrease in conduction time during the supernormal period was very marked in this experiment and obviously no local decrease of latency can explain the effect. Observations on the conduction rate between two points (116–202.5 cm. apart, last four experiments in the table) further showed that the decreased conduction time was due to an increased conduction rate, and could be explained in no other way.

f. *After-potential.* The essential facts regarding the development of the power to exhibit after-potential paralleling that of the power to exhibit supernormality have already been published (8). As an additional illustration of the known facts, after-potential records taken before and after the irritability observations plotted in figure 1 are set into the figure. Before the irritability determinations, a small after-potential was manifest even though no supernormal irritability could be found at this stage of the experiment. At the end of the experiment the after-potential was perhaps five times as high and lasted twice as long as before; at this stage the supernormal irritability lasted longer than the negative after-potential record, probably because the recorded negativity was prematurely cut off by the after-potential developing under the grid lead.

II. *Effects of veratrinization.* a. *Irritability.* Veratrine has been found by all workers (12, 13, 15) to increase the irritability of nerve in early poisoning. In view of the prolonged supernormal period of such nerves, it seemed possible however that the increased irritability might be apparent only, due to introduction of each shock into the supernormal period following the preceding response. The rate of stimulation used in the irritability determinations previously reported from this laboratory was not recorded but was probably about 15 shocks per minute; as it was found in the same investigation that in veratrinized nerve the supernormal period following a response may last 2 or 3 seconds, observations of resting irritability made with less frequent stimulation seemed desirable. In view of the relation between after-potential and supernormality, the recent evidence that the veratrine after-potential may last a number of minutes (6) also indicated the desirability of such experiments.

Accordingly, the strength of shock necessary to produce a threshold response was determined with shocks at a rate of 6 to 16 per minute; after a complete rest of 2 to 30 minutes, the nerve was again stimulated by a single shock of this strength. In veratrinized as in normal nerve, the height of response was the same after this long interval as after the interval of a few seconds; if the interval was reduced to 1 second, the degree of

supernormality was sometimes sufficient to increase considerably the apparent resting irritability of a veratrinized nerve, but never that of a normal nerve. Determinations of the resting irritability of nerve before and after veratrinization, therefore, if made with stimulation rates of 1 per second, give a spuriously great increase in this nerve function; but with a rate of 15 or fewer stimulations per minute, give the same and undoubtedly the true increase whatever the rate. The resting irritability of three nerves determined by stimulation at 2 to 5 minute intervals was found to be increased 12, 20 and 23 per cent respectively by veratrinization with 1/50,000 veratrine hydrochloride solution carefully painted on the nerve with a minimum change in its contact with the electrodes. In figure 4 is plotted one of a number of experiments with a stimulation rate of 15 per minute, giving similar increases of irritability (11.5 per cent in the experiment plotted). These results establish beyond doubt the fact that veratrine actually increases the resting irritability, and confirm the results previously published.

The fact that veratrinization increases irritability while at the same time increasing the degree of supernormality (see below) is the important experimental finding in connection with the problem under investigation; this increase of irritability, however, is characteristic of early poisoning only, and as the irritability may fall to normal or below while the after-potential is still large, the increase may be overlooked under certain conditions.

b. *Relatively refractory period.* The experiment plotted in figure 4 is typical in showing that the relatively refractory period of an unpoisoned nerve, shortened by the development of the supernormal period (curve 1), does not undergo great further shortening with veratrinization of the nerve (curve 2); in many cases there seems to be no further shortening at all, in confirmation of the already published observations of unchanged relatively refractory period in early veratrinization.

c. *Supernormal period.* During the supernormal period, however, the irritability of the veratrinized nerve in general rises more than does that of the unpoisoned nerve for a given increase in interval between the conditioning and testing shocks; since the interval during which irritability rises is the same in the two conditions, the irritability reaches a higher level in the veratrinized nerve. 110 to 125 per cent of the resting irritability is the typical maximal value of supernormality in veratrinized, as contrasted with 105 to 110 per cent in unpoisoned nerve; in one veratrinized nerve, maximal supernormality of 151 per cent of the resting irritability was observed. In the experiment plotted in figure 4, the maximal supernormal irritability before veratrinization was 109 per cent (curve 1) of its resting level; after veratrinization, 114 per cent (curve 2) of the new resting level. As the new resting level was 11.5 per cent higher than the normal

level, the maximal supernormal irritability in the veratrinized nerve was 27 per cent above the resting normal level (curve 3).

It has not been possible to find any correlation, either direct or inverse, between the increase of resting irritability and the increased degree of supernormality resulting from veratrinization.

The gradual rise of irritability to its maximum is followed in veratrinized as in unpoisoned nerve by an even more gradual fall. The initial rate of

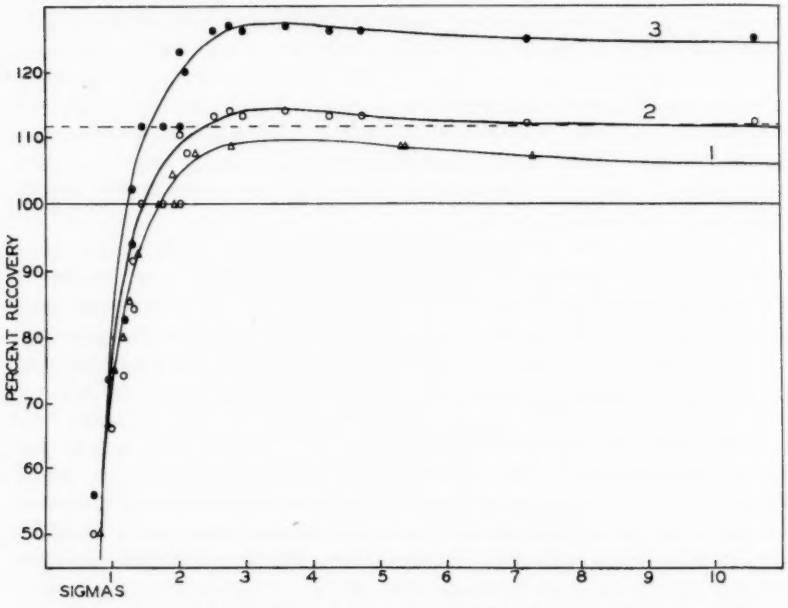

Fig. 4. Increased irritability and supernormality with veratrinization. Green frog sciatic nerve. Conduction distance = 6 mm. for testing shock, 15 mm. for conditioning shock. 1, recovery curve of unpoisoned nerve 1 to 2 hours after isolation. Veratrine hydrochloride (1/250,000) then painted on; 1 to 2 hours later, irritability 111.5 per cent of pre-veratrinization level. 2, recovery curve plotted with new irritability level as 100 per cent; 3, with new irritability level as 111.5 per cent (dotted line). Abscissae = interval between conditioning and testing shocks. Temp. = 24°C. 5/30/33.

fall is probably very similar in the two conditions (fig. 4), but it seems from the long durations of the supernormal period already observed in veratrinized nerves (2–3 seconds) that the later portions must be very much drawn out; and sometimes, as in the nerve used in the experiment plotted in figure 3, the irritability remains practically maximal for much longer intervals than in unpoisoned nerve.

d. *Recovery of height of response.* Re-investigation of the effect of veratrinization on this function seemed superfluous in connection with the present problem, in view of the facts already published and those outlined above.

e. *Conduction rate.* Veratrinization tends to lower the conduction rate in green frog nerves (12); that of the bullfrog nerves used in this investigation seemed rather more susceptible to this effect of the poison (last two experiments of the table), possibly because finer portions of the nerve were included in the preparation. The increase in conduction rate during the supernormal period in veratrinized nerves has already been discussed in conjunction with the same phenomenon in unpoisoned nerves.

f. *After-potential.* The only point which seems to require mention in connection with the after-potential of veratrinized nerve is the position of its crest in relation to that of the irritability crest. In unpoisoned nerve, the after-potential reaches its maximum value after a shorter interval following a shock than does the irritability; in veratrinized nerve the prolonged rise of the after-potential ordinarily brings the maximal after-potential at least as late as the region of maximal irritability in normal nerve, and the start of maximal irritability in veratrinized nerve (fig. 3). However, in the latter condition, the irritability remains practically maximal over a range so considerable that even the most delayed after-potential crests would probably not fall beyond it.

Discussion. The evidence just presented shows conclusively that the phenomenon of supernormality does not depend upon depression of the level of resting irritability, but rather upon modification of the recovery process. The nature of this modification is manifest from consideration of the simplest known case, spontaneous development of supernormality in isolated nerve. This change in the nerve does not affect the duration of the period of rise of irritability, which begins at exactly the same interval after the shock (unchanged absolutely refractory period) and continues for the same length of time as before. Neither is there any alteration of the general character of the rise, which follows a perfectly smooth curve at all stages of development of supernormality. The modification of the recovery process consists primarily in an increased rate of rise of irritability during the latter part of the rise; since the duration of the period of rising irritability is unchanged, the faster rise results in a greater degree of irritability than before; the supernormal irritability thus produced is reduced just to the original level of resting irritability during the subsequent prolonged phase of declining irritability.

When supernormality is developed by means of veratrine or increased hydrogen ion concentration, this simple picture is complicated by a simultaneous change in the level of resting irritability, which is lowered by veratrine and raised by acidity. The change is obviously not an integral

part of the development of supernormality, and when the new level of resting irritability is accordingly adopted as the standard of reference, the modification of the recovery process involved in the development of supernormality in each case reduces to the terms describing the simplest case.

A further complication is introduced if increased bivalent cation (Ca^{++}) concentration is classified as an agent favoring supernormality; such a classification seems justified by the prolongation of the supernormal period by this agent, even though it does not increase the maximal degree of supernormality. The additional complication in this case is that the period during which irritability rises is shortened; it begins at the same time as before, but does not last so long. Consequently here again, if the new depressed level of resting irritability be taken as the standard of reference, the change in the recovery process may be regarded as an increased rate of rise of irritability, together with a prolongation of the period of declining irritability.

As the rate of recovery of irritability increases with the development of supernormality, the rate of recovery of height and that of conduction velocity increase also, apparently as dependent variables. Regarding the height, there might be some question as to this view of its rôle, since the height does not become supernormal with the irritability; but this must be due to a further limitation set on it by a third factor. The intimate connection between rate of rise of height and that of irritability is clear, and of the two, rate of rise of irritability must be the controlling factor since it is unaffected by the cessation of the rise of height on the attainment of normal height. The subordinate position of recovery of height is suggested also by the behavior of the conduction velocity in the refractory and supernormal period. For theoretical reasons, this has been considered as controlled by both irritability and height, but the above experimental results indicate that for equivalent changes in irritability, the depression of conduction rate during the relatively refractory period when both irritability and height are decreased is no greater than its enhancement during the supernormal period when the irritability but not the height is increased. Insofar as such a quantitative comparison between the two situations may be justified, it suggests that at least in the latter part of the refractory period, the depression of height of response is relatively unimportant.

The rate of rise of irritability, as well as the subsequent fall of irritability to normal, is certainly related in some way to the after-potential process. That there is a relationship between supernormality and after-potential has been obvious for some time from the similarity in the behavior of the two under various conditions; and if the essence of supernormality is a change in the rate of rise of irritability, it may be that the rôle of the after-potential process is the determination of the rate of rise and likewise that

of subsequent fall of irritability. That the after-potential process and not the supernormality is primary has always seemed likely, since the after-potential may be increased (e.g., by excess Ca^{++}) without an accompanying increased degree of supernormality. Until more is known of the nature of the after-potential process, it will not be possible to indicate how it brings about its effects on irritability.

The duration of the period of rise of irritability is evidently independent to some extent of the rate of rise of irritability and of the resting level of irritability. A priori one might expect this period to end when a certain degree of irritability had been attained, but this is obviously not the limiting factor. Nor can the duration of the spike potential be the sole limiting factor (9), since the period of rising irritability, though more constant than the relatively refractory period, still is less constant than the duration of the spike potential. For example, excess of univalent or bivalent cations affects the period of rising irritability, but not the spike duration. Nor can the after-potential process be the sole limiting factor, for the after-potential crest usually does not coincide with the irritability crest. Nevertheless since agents which prolong the duration of the rise of irritability also retard its rate of rise, and (in agreement with the relation of the rate of rise to the after-potential) likewise decrease the after-potential, there must be some indirect relationship between the after-potential and the duration of the rise of irritability. The known facts do not justify a more positive statement regarding the duration of the period of rising irritability than that it may be determined by a combination of the spike potential and after-potential processes, and possibly by some other factors as well.

The period of rising irritability rather than the relatively refractory period has been adopted as the physiological unit in the above discussion. Clearly, the experimental findings regarding the recovery process might alternatively be formulated in terms of the relatively refractory period, and such a formulation is attractive because this period always marks the attainment of normal irritability, height and conduction rate. However, it offers no explanation of the constancy of the period of rising irritability when the relatively refractory period varies, nor of the difference in approach of the rising irritability curve to the level of resting irritability in the absence and in the presence of supernormality, nor of the smooth, continuous passage of the rising irritability curve across the level of resting irritability in the latter condition.

SUMMARY

In a freshly isolated frog nerve, the relatively refractory period lasts 7 to 10σ after a response (25–29°C.) and is not followed by a period of supernormal irritability. On standing ($\frac{1}{2}$–$1\frac{1}{2}$ hour), the nerve gradually develops the capacity to exhibit supernormality. Simultaneously the

relatively refractory period shortens to 1.5 or 2.5σ and maximal supernormal irritability now falls where the relatively refractory period ended in the fresh nerve. Normal height of action potential and normal conduction rate are always regained after the same interval as normal irritability; the height rises no farther, but the conduction rate goes through a supernormal period corresponding to that of irritability. The maximal degree of supernormality of irritability is usually between 105 and 110 per cent of the resting value; the supernormality of conduction rate is less than that of irritability. The normal level of irritability, height of response and rate of conduction do not change when the nerve acquires the ability to manifest supernormality.

Veratrinization increases the resting level of irritability 10 to 25 per cent; it also increases and prolongs supernormality. Maximal supernormality is attained after practically the same interval following a shock as in untreated nerve. The relatively refractory period is not further shortened greatly.

The phenomenon of supernormality therefore depends not upon depression of the normal level of irritability, but upon a modification of the recovery process. This modification consists essentially of an increased rate of rise of irritability during the latter part of the rise, with a subsequent fall of irritability; it may or may not be accompanied by a change in the resting level of irritability and in the duration of the rise of irritability.

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THE EFFECT OF CONTINUED ORAL ADMINISTRATION OF HISTAMINASE AND PANCREATIN ON GASTRIC SECRETION

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The fact that histaminase under certain circumstances inactivates histamine suggested to us the possibility that it might have some effect upon the acidity of the gastric contents when administered orally. The observation of Melli and Boggian (1) that pancreatin, after from five to ten days of oral doses of from three to four grams, brought about in humans a reduction in the gastric acidity, sometimes even to complete achlorhydria, made us think that pancreatin may contain histaminase and that this substance might be responsible for the effects secured. With these views we conducted a series of experiments both with histaminase and with pancreatin.

METHODS. Histaminase was prepared from fresh hog kidney, following the method of C. H. Best and E. W. McHenry (2). The pancreatin used was the U. S. P. preparation of Eli Lilly & Company.

The inactivating effect of the histaminase powder was tested after 24 hours' incubation at 37° with histamine in phosphate buffered solution at pH 7. Both diazo reactions and biological tests were used. We found the diazo reaction very unsatisfactory due to the interference of color, which made it difficult to interpret the presence of histamine in the specimens mixed with "kidney powder" or pancreatin. It seemed clear, though, that the pancreatin either alone or incubated with histamine gave a positive reaction for the latter substance.

For the biological tests, intravenous injections were given to an etherized vagotomized cat and the blood pressure changes were taken as indicative of the presence or absence of histamine. The injection of 6.6 mgm. of "kidney powder" in 1 cc. of buffer produced no change in blood pressure and neither did the "kidney powder" when incubated with histamine, our results indicating that 500 mgm. of "kidney powder" completely inactivated 1 mgm. of histamine. The injection of 6.6 mgm. of pancreatin alone, and of the same amount of pancreatin incubated with histamine (500 mgm. pancreatin to 1 mgm. histamine) brought about a marked lowering of the blood pressure, confirming the results obtained with the diazo reaction. In

spite of the findings in regard to pancreatin, the experiments which were already in progress concerning its effect on gastric acidity were continued to their termination.

An experiment with histaminase was carried out on a normal human with the following dosage: 2 grams daily in gelatine capsules before retiring, for a period of 21 days. Pancreatin was administered to two normal humans and one dog in the following way: in the humans, 2 grams daily in gelatine capsules for 16 days and for another period of 16 days, 6 grams daily,—in the latter case in divided doses shortly before meals; in the dog pancreatin was given in daily doses of 10 grams for a period of 30 days.

The gastric contents were withdrawn under fasting conditions, half hour and one hour after an Ewald meal in the humans, and after stimulation with meat extract solution in the dog. Titrations of free, combined and total acids were carried out in these specimens. Tests were performed before, during and after the administration of the "kidney powder" and the pancreatin.

Results. Administration of histaminase did not result in any noticeable change in the acidity of the gastric contents. The curves obtained before these substances were given are comparable with those obtained after and during their continued administration. We may mention the fact that no objective or subjective symptoms developed in our subjects in the course of the experiment.

Pancreatin also had no effect on the acidity of the gastric contents. As we have mentioned above, we were originally motivated in repeating the work of Melli and Boggian to see whether their positive results with pancreatin could be ascribed to histaminase. We were, however, unable to confirm their results.

Discussion. While our work was in progress we came across an article published by Atkinson and Ivy (3) showing that the intravenous administration of histaminase in dogs does not affect the gastric secretory response to a meal or an injection of histamine. The authors also mention the fact that histaminase fed with the meal produces no effect. They feel that the failure of histaminase to act upon the gastric secretion is due to the fact that histamine is absorbed before being inactivated by the histaminase.

Our findings indicate that even the long continued administration of histaminase, when given by mouth, fails to produce any effects on the gastric secretion. In our experiments we think that the acidity of the stomach may have destroyed a good part of the histaminase contained in the kidney powder. Best and McHenry have shown that, in vitro, histaminase which has been incubated at a pH of 2.5 loses and does not recover its activity. Another possible cause of failure may have been the effect of the digestive enzymes upon histaminase, which, as far as we know, has not been studied.

CONCLUSIONS

1. Daily oral administration of histaminase powder for a period of 21 days has no effect upon the acidity of the gastric juice.

2. Continued administration of pancreatin U. S. P. Lilly in fractional doses for long periods does not modify the acidity of the gastric juice.

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